# METHOD OF REPRESSING FLOWERING IN A PLANT

## Field of Invention

The present invention relates in part to a method of reducing or substantially preventing flowering in a plant, the method comprising expressing a newly identified flowering repressor sequence in the plant to produce a polypeptide which represses flowering in said plant. More particularly the invention relates amongst other things to the provision of vectors, cells or transgenic plants comprising said sequences or related sequences and uses thereof, and a promoter for up-regulating gene expression in the apex and leaves during long day treatment. In certain aspects of the invention, the plant may be a perennial or biennial plant; in certain further aspects of the invention, the plant may be a monocotyledonous plant.

## Background to the Invention

The life cycle of flowering plants in general can be divided into three growth phases: vegetative, inflorescence, and floral (Poethig 1990). In the vegetative phase, the shoot apical meristem (SAM) generates leaves that will later ensure the resources necessary to produce fertile offspring. Upon receiving the appropriate environmental and developmental signals, the plant switches to floral, or reproductive, growth and the SAM enters the inflorescence phase ($I_1$) and gives rise to an inflorescence with flower primordia. During this phase, the fate of the SAM and the secondary shoots that arise in the axils of the leaves is determined by a set of meristem identity genes, some of which prevent and some of which promote the development of floral meristems. Two basic types of inflorescences have been identified in plants: determinate and indeterminate (Weberling, 1989). In determinate species, such as ryegrass, the SAM eventually produces floral organs and the production of meristems is terminated with a flower. The SAM of indeterminate species is not converted to a floral identity and will therefore only produce floral meristems from its periphery, resulting in a continuous growth pattern.

The regulation of meristem identity and floral transition has been investigated in a number of dicotyledonous plants including Arabidopsis, *Antirrhinum,* tomato, and tobacco. However; in agronomically important seed crops such as wheat, barley, rice, forage grasses, and other monocotyledonous plants, information on how floral transition is controlled is still limited. The

present inventors have undertaken a molecular investigation of the regulation of meristem identity and the control of floral transition in perennial ryegrass *(Lolium perenne)*, a cool-season perennial forage grass native to Europe, temperate Asia, and North Africa.

There are several reasons for such an investigation. Firstly the production of culm (stem) and seed head (inflorescence) formation decreases the feeding value of forage grasses. The leaf blades are more digestible, richer in crude protein and poorer in cell-wall constituents than sheaths and culms (Deinum and Dirvan, 1975; Wilman et al., 1976). The ageing of grasses is associated with an increase in lignification and a decrease in digestibility, which is markedly higher for the stems than for the leaves (Delagarde et al., 2000). Feeding trials on cows have documented that increasing the digestibility of forage grass leads to a daily increase in feed uptake and milk production (Oba and Allen, 1999). Secondly, maintenance of a vegetative forage grass requires a frequent mechanical defoliation system, which is both costly and time consuming. Too intensive defoliation can severely decrease the photosynthetic capacity of the plant and in the worst case destroy the regeneration capacity. Thirdly, flowering in many plants is associated with an uncontrollable gene flow from cultivated to wild species via the active spread of pollen. Fourthly, flowering in many perennial plants is also associated with an exposure of grass pollen allergens. A grass cultivar with an extended vegetative growth associated with decreased or even eliminated inflorescence production would thus be agronomically attractive.

In terms of plant development, the aerial parts of ryegrass are produced by the apex positioned on the base crown a few millimetres above the ground and surrounded by developing leaves (Fig. 1A). During vegetative growth the apical meristem generates lateral meristems initially recognised as semicircular ridges along the main axis. These become the leaf primordia. This morphological pattern does not change until the apex has been induced to flower by elevated temperatures and increasing day length. Flowering in perennial ryegrass is induced by a vernalization period of 12 to 14 weeks below 5°C followed by secondary induction with long-day photoperiods (generally, more daylight hours than dark hours and, in particular, LD, 16 h of light, 8 h of darkness) and temperatures above 15 to 20°C. Upon transition to reproductive growth, the apical meristem and later also the lateral meristems start to expand and eventually turn into groups of inflorescences (spikelets), each containing three to 10 floral meristems. The flowers of the

ryegrass inflorescence are arranged in a cymose, always terminating apical growth with the production of a terminal flower. In this way ryegrass represents a determinate plant architecture also seen and described at the molecular level in dicot plants such as tobacco (Amaya et al., 1999) and tomato (Pnueli et al., 1998). In contrast to plants such as Arabidopsis and *Antirrhinum*, ryegrass has a determinate (cymose) inflorescence. The *TERMINAL FLOWER 1 (TFL1)* gene of Arabidopsis and its homolog *CENTRORADIALIS (CEN)* in *Antirrhinum* have been identified as a group of genes that specify an indeterminate identity of inflorescence meristems. Mutations in *TFL1/CEN* result in the conversion of the inflorescence into a terminal flower (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992;). In addition to its effect on meristem fate, *TFL1* also extends the vegetative phase of Arabidopsis (Shanon and Meeks-Wagner, 1991, Ratcliff *et al.*, 1998), but *CEN* does not seem to have a flowering time role in *Antirrhinum* (Bradley et al., 1996). CEN and TFL1 proteins have sequence similarity with mammalian phosphatidylethanolamine-binding proteins (PEBPs). The *FLOWERING LOCUS T (FT)* gene also belongs to the family of plant PEBP genes, but has been shown to play an opposite role to *TFL1* in mediating flower inducing signals in Arabidopsis (Kardailsky et al., 1999; Kobayashi et al., 1999). Therefore the family of plant PEBP genes comprise a number of homologues proteins with different properties in relation to floral control. These differences are further revealed by the expression of different plant PEBPs from a constitutive promoter in different plant species. Expression of the *TFL1* gene in tobacco from the 35S CaMV constitutive promoter, for example, does not affect the flowering time and does not affect the plant architecture of tobacco (Amaya *et al.*, 1999).

The differences in gene function are also reflected in the different expression patterns by plant PEBPs. In *Arabidopsis* and *Antirrhinum, TFL1/CEN* is expressed in the centre of the SAM. Upon transition from vegetative to reproductive growth, the expression of these genes increases (Bradley *et al.*, 1996, 1997). Expression of the floral meristem identity genes such as *LFY, AP1* and *CAL* is also increased in the SAM upon transition to reproductive growth, but the expression is confined to the developing flowers (Mandel et al., 1992; Bradley et al., 1997; Ratcliffe et al., 1999). In tobacco the *CET2/CET4* genes are mainly expressed in the axillary meristems and not in the SAM (Amaya et al., 1999).

**Summary of the Invention**

The present invention is based on investigations on the mechanism underlying flowering control and plant architecture in a widely distributed, agronomically important monocot crop plant. The present inventors had previously isolated a gene from perennial ryegrass, *LpTFL1*, which shows homology to the group of plant PEBPs, and demonstrated its role in determining plant architecture and effectively repressing the vegetative-reproductive phase transition in plants. The inventors have now transformed *Arabidopsis thaliana,* red fescue (*Festuca rubra* L.), and ryegrass (*Lolium perenne* L) with *LpTFL1* and the results indicate that *LpTFL1* is a repressor of flowering with a unique phenotypic effect not reported before. Overexpression of *LpTFL1* in Arabidopsis, red fescue, and ryegrass results in a dramatic extension of the vegetative-inflorescence phase and a lateral branching in *Arabidopsis* that is consequently more extreme compared with overexpression of *TFL1* in Arabidopsis. In addition, the results illustrate that *LpTFL1* is capable of repressing flowering in perennial plants in the first year of growth and also in subsequent years. Repressors capable of preventing flowering and persisting to subsequent years have not hitherto been identified. The present inventors have also isolated the promoter of *LpTFL1* and determined its potential use for up-regulating gene expression during long day flower induction.

Therefore, it is an object of the present invention to provide an isolated flowering repressor gene, the encoded protein, and its flowering related promoter, from plants.

Thus, in a first aspect, the present invention provides a method of significantly reducing or substantially preventing flowering in a perennial or biennial plant, the method comprising expressing a polypeptide from an isolated polynucleotide fragment comprising a nucleotide sequence as shown in Figure 2, or a fragment, derivative, or homologue thereof, in a perennial or biennial plant. The nucleotide sequence represents a gene sequence isolated from the ryegrass *Lolium perenne* which is hereinafter referred to as "LpTFL1". Preferably the plant is a perennial.

In a second aspect, the present invention provides a method of significantly reducing or substantially preventing flowering in a perennial or biennial plant, the method comprising expressing an isolated polypeptide having an amino acid sequence as shown in Figure 4, or a functionally active fragment, derivative or homologue thereof. The isolated polypeptide is encoded

by the isolated polynucleotide fragment as shown in Figure 2, or a fragment, derivative, or homologue thereof. Preferably the plant is a perennial.

The term "polynucleotide fragment" as used herein refers to a chain of nucleotides such as deoxyribose nucleic acid (DNA), oligonucleotides and transcription products thereof, such as RNA, mRNA, etc. The term may also be used interchangeably herein with the terms "polynucleotide", "DNA coding sequence", "gene", "genetic material", "gene sequence" and "genetic sequence".

The polynucleotide fragment can be isolated in the sense that it is substantially free of biological material with which the whole genome is normally associated in vivo. The isolated polynucleotide fragment may be cloned to provide a recombinant molecule comprising the polynucleotide fragment. Thus, "polynucleotide fragment" includes double and single stranded DNA, RNA, oligonucleotide and polynucleotide sequences derived therefrom, for example, sub-sequences (also referred to herein as sub-fragments) of said fragment and which are of any desirable length. Where a nucleic acid is single stranded then both a given strand and a sequence complementary thereto is within the scope of the present invention.

The polynucleotide fragment may be expressed in order to provide an expression product. In general, the term "expression product" refers to both transcription and translation products of said polynucleotide fragments. When the expression product is a "polypeptide" (i.e. a chain or sequence of amino acids displaying a biological activity substantially similar to the biological activity of an essential protein, it does not refer to a specific length of the product as such. Thus, the skilled addressee will appreciate that "polypeptide" encompasses inter alia peptides, polypeptides and proteins. The polypeptide if required, can be modified in vivo and/or in vitro, for example by glycosylation, amidation, carboxylation, phosphorylation and/or post-translation cleavage.

As is well known in the art, the degeneracy of the genetic code permits substitution of bases in a codon resulting in a different codon which is still capable of coding for the same amino acid, e.g. the codon for amino acid glutamic acid is both GAT and GAA. Consequently, it is clear that for the expression of polypeptides with the amino acid sequence shown in Figure 4, or fragments thereof, use can be made of other nucleic acid sequences with such an alternative codon composition different from the nucleic acid sequence shown in Figure 2. These are referred to herein as "derivatives".

The terms "homologues" or "homologous" as used herein refers to nucleotide sequences of polynucleotide fragments of the present invention which have 65% identity or above with the sequence disclosed herein, such as 66%, 68%, 70%, 75%, 80%, 83%, 86%, 88%, 90%, 92%, 95%, 97% or 99% identity. Such polynucleotide fragments may include, but are not limited to, known sequences which have not previously been identified as flowering repressors, such as *FDR1* (85% identity) from rice, *CET1* (66% identity), *CET2* (72% identity), *CET4* (71% identity) from tobacco, *SP* (70% identity) from tomato, and *BnTFL1-1* (68% identity), *BnTFL1-3* (68% identity) from rapeseed, or may include sequences which are only known to delay flowering to some extent, such as *FDR2* (87% identity) and *RCN2* from rice,

The term "identity" with respect to nucleotide sequences is defined as the percentage of nucleotides in a polynucleotide sequence which are identical to the nucleotides in the sequence disclosed herein after alignment as determined by using sequence analysis programs. Programs which are used for database searching and sequence alignment and comparison, for example, from the Wisconsin Package Version 10.2, such as BLAST, FASTA, PILEUP, FINDPATTERNS or the like (GCG, Madison, WI) or public available sequence databases such as GenBank, EMBL, Swiss-Prot and PIR or private sequence databases such as PhytoSeq (Incyte Pharmaceuticals, Palo Alto, CA) may be used to determine sequence identity, Alignment for sequence of comparison may be conducted by the local homology algorithm of Smith and Waterman (1981: Adv. Appl. Math., 2:482), by the homology alignment algorithm of Needleman and Wunsch (1970: J. Mol. Biol., 48:443), by the search for similarity method of Pearson and Lipman (1988: Proc. Natl. Acad. Sci. USA., 85: 2444), by computerized implementations of these algorithms.

Using the information provided by the present invention, isolated polynucleotide fragments similar to the LpTFL1 polynucleotide fragments disclosed herein for use in the methods of the present invention may now be obtained from any plant source using standard methods, for example, by employing consensus oligonucleotides and PCR. By "similar" is meant an isolated polynucleotide fragment comprising a nucleotide sequence which is capable of hybridising to a sequence which is complementary to the nucleotide sequence of the inventive polynucleotide fragment. The stringency of the hybridisation is used to determine the degree of similarity between two sequences. Normally, stringent conditions are selected to be about 5°C to 20°C lower than the

thermal melting point ($T_m$) for the specific sequence at a defined ionic strength and pH. The $T_m$ is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridise to a perfectly matched sequence (probe).

In the case that the similar and inventive sequences are mixed together and denatured simultaneously, the Tm values of the sequences are preferably within 10°C of each other. More preferably hybridisation may be performed under stringent conditions, with either the similar or inventive DNA preferably being supported. Thus for example either a denatured similar or inventive sequence is preferably first bound to a support and hybridisation may be effected for a specified period of time at a temperature of between 50 and 70°C in double strength SSC (2 x NaCl 17.5g/l and sodium citrate (SC) at 8.8g/l) buffered saline containing 0.1% sodium dodecyl sulphate (SDS) followed by rinsing of the support at the same temperature but with a buffer having a reduced SSC concentration. Depending upon the degree of stringency required, and thus the degree of similarity of the sequences, such reduced concentration buffers are typically single strength SSC containing 0.1% SDS, half strength SSC containing 0.1% SDS and one tenth strength SSC containing 0.1% SDS.

Sequences having the greatest degree of similarity are those the hybridisation of which is least affected by washing in buffers of reduced concentration. It is most preferred that the similar and inventive sequences are so similar that the hybridisation between them is substantially unaffected by washing or incubation at high stringency, for example, in one tenth strength sodium citrate buffer containing 0.1% SDS. These similar polynucleotide fragments from plants other than ryegrass are also encompassed by the term "homologues".

Therefore, the present invention also provides a method of significantly reducing or substantially preventing flowering in a perennial or biennial plant, the method comprising expressing a polypeptide from an isolated polynucleotide fragment comprising a similar nucleotide sequence, or fragments thereof, from other plants which are complementary to the one which hybridises under stringent or moderately stringent conditions with the nucleotide sequences of the isolated polynucleotide fragments, or fragments thereof, disclosed herein. The isolated polynucleotide fragments comprising a similar nucleotide sequence employed in the present invention may be isolated from any plant including monocots and dicots and in particular

agriculturally important plant species, including but not limited to, crops such as those belonging to the grass family of *Poaceae*, and also soybean, potato, oilseed rape, sunflower, alfalfa, sugar cane and cotton; or herbs such as anise, basil, bay laurel, caper, caraway, cayenne pepper, celery, chervil, chives, coriander, dill, fennel, garlic, horseradish, leeks, lemon balm, liquorice, marjoram, mint, oregano, parsley, rosemary, sesame, tarragon and thyme; or fruits and vegetables, such as banana, blackberry, blueberry, strawberry, and raspberry, carrot, coffee, eggplant, grapes, honeydew, mango, onion, papaya, peas, peppers, pineapple, rosaceous fruits (such as apple, peach, pear, cherry and plum) and vegetable brassicas (such as brussel sprouts). The homologues may also be derived from woody species, such as eucalyptus, oak, pine and poplar.

The invention also provides a method of significantly reducing or substantially preventing flowering in a perennial or biennial plant, the method comprising expressing a polypeptide from an isolated polynucleotide fragment comprising a similar nucleotide sequence which is synthetic or artificial and is complementary to one which hybridises under stringent or moderately stringent conditions with the above disclosed nucleotide sequences, or fragments thereof.

The inventors have identified the genomic sequence corresponding to the cDNA sequence of Figure 2, which is illustrated in Figure 3. The coding sequence, including introns and exons, of LpTFL1 is from bases 1 to 912. Therefore, the present invention further provides an isolated polynucleotide fragment comprising the nucleotide sequence of bases 1 to 912 or bases -78 to 1242 of Figure 3. Although base 1242 corresponds to the end of the cDNA sequence, it is thought that that sequence from base 1243 to base 1624 may comprise a polyadenylation signal. Therefore, the isolated polynucleotide fragments of the present invention may further comprise bases 1243 to 1624 in conjunction with bases 1 to 1242. In addition, the present invention provides a method of reducing or substantially preventing flowering in a perennial or biennial plant, the method comprising expressing a polypeptide from an isolated polynucleotide fragment comprising the nucleotide sequence of bases selected from the group consisting of bases 1 to 912, bases 1 to 1624, bases -78 to 912, bases -78 to 1242, and bases -78 to 1624, all of Figure 3.

The inventors have also isolated a region upstream from the start codon that comprises the native promoter for the LpTFL1 gene in ryegrass (bases -3600 to -1). Therefore, also provided in

the present invention is a polynucleotide fragment which comprises a promoter with the nucleotide sequence of bases -3600 to -1 of Figure 3.

The inventors determined the function of the *LpTFL1* promoter by characterising the expression of *LpTFL1* mRNA in ryegrass. The results showed that *LpTFL1* is expressed in the apex of ryegrass at the vegetative stage. In contrast to *TFL1* in *Arabidopsis,* the expression of *LpTFL1* mRNA not only increases in the apex but also in the leaves (more than 25 fold) upon transition to reproductive growth. The increase in *LpTFL1* mRNA expression in leaves seems to be stimulated by flowering induction i.e. long day treatment and temperature increase. Therefore, in another aspect, the present invention provides an isolated polynucleotide fragment having a nucleotide sequence of bases -3600 to -1 as shown in Figure 3, or a fragment or derivative thereof, for up-regulating gene expression in the apex and leaves of a perennial or biennial plant during conditions that lead to flowering.

Flowering induction and conditions that lead to flowering are herein defined as a period of 8 to 18 weeks, in particular 10 to 16 weeks and in particular 12 to 14 weeks below 5°C followed by secondary induction with long-day photoperiods which are generally more daylight hours than dark hours and, in particular, 13 h of light and 11 h of darkness, 14 h of light and 10 h of darkness, 15 h of light and 9 h of darkness, or 16 h of light and 8 h of darkness, and/or temperatures above 15°C, such as 16°C, 17°C, 18°C, 19°C, 20°C or above.

It will be understood that for the particular polypeptides embraced herein, variations can be made to the polypeptides without substantially altering their function. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence and are referred to as "derivatives" when referred to together with polypeptides. All such polypeptide derivatives showing the recognised physiological activity are included within the scope of the invention. For example, for the purpose of the present invention conservative replacements may be made between amino acids within the following groups:

(I) Alanine, serine, threonine;

(II) Glutamic acid and aspartic acid;

(III) Arginine and leucine;

(IV) Asparagine and glutamine;

(V) Isoleucine, leucine and valine;

(VI) Phenylalanine, tyrosine and tryptophan.

Also encompassed are synthetic amino acids incorporated into the polypeptides of the present invention, either by way of addition or substitution of existing amino acids.

Polypeptides, or fragments or derivatives thereof, modified as hereinbefore described, and which retain the physiological activity of the original, full-length peptide are referred to herein as "functional variants" or "functionally active variants". Moreover, recombinant DNA technology may be used to prepare nucleic acid sequences encoding the various derivatives outlined above.

The amino acid sequence or sequence motifs of the present invention may be used in homology searches in protein databases to find LpTFL1 related proteins from other plant species. Therefore, the present invention also provides a method of significantly reducing or substantially preventing flowering in a plant, the method comprising expressing polypeptide sequences homologous to the polypeptide sequence disclosed herein from another plant. When referred to together with polypeptides, the term "homologue" refers to polypeptide sequences which have 69% identity or above with the sequence disclosed herein, such as 70%, 72%, 75%, 80%, 83%, 87%, 90%, 92%, 95%, 97% or 99% identity. The polypeptide sequences may be isolated from a plant such as a monocot or a dicot. The plant may further be an annual, a biennial or a perennial. Such polypeptide sequences may include, but are not limited to, known sequences which have not previously been identified as flowering repressors, such as FDR1 (86% identity) from rice, CET1 (68% identity), CET2 (72% identity), and CET4 (72% identity) from tobacco, SP (71% identity) from tomato, and BnTFL1-1 (70% identity) and BnTFL1-3 (70% identity) from rapeseed; or sequences which are known to be partially effective repressors of flowering, such as FDR2 (91% identity) andRCN2 from riceThe term "identity" with respect to polypeptide sequences is defined as the percentage of amino acids in an polypeptide sequence which are identical to the amino acids in the polypeptide sequence disclosed herein after alignment as determined by using sequence analysis programs. Programs which are used for database searching and sequence alignment and comparison, for example, from the Wisconsin Package Version 10.2, such as BLAST, FASTA, PILEUP, FINDPATTERNS or the like (GCG, Madison, WI) or public available sequence databases

such as GenBank, EMBL, Swiss-Prot and PIR or private sequence databases such as PhytoSeq (Incyte Pharmaceuticals, Palo Alto, CA) may be used to determine sequence identity. Alignment for sequences of comparison may be conducted by the local homology algorithm of Smith and Waterman (1981: Adv. Appl. Math., 2:482), by the homology alignment algorithm of Needleman and Wunsch (1970: J. Mol. Biol., 48:443), by the search for similarity method of Pearson and Lipman (1988: Proc. Natl. Acad. Sci. USA., 85: 2444), by computerized implementations of these algorithms.

The terms "homologue" or "homologous" may also refer to polypeptide sequences, which are estimated to be clustered together with the polypeptide disclosed herein after a cladistic analysis of two to several homologous polypeptide sequences as determined by sequence analysis programs for multiple sequence alignments available, for example ClustalW (Tompson et. al. 1994: Nucleic Acids Res., 22:4673) or PILEUP which is included in the Wisconsin Package version 10.2 (GCG, Madison, WI) or the like. The cladistic analysis of sequences for comparison may be conducted by using different values for the ClustalW program parameters such as, gap open penalty, gap extension penalty, gap separation penalty, and protein weight matrix. The values for these parameters may be; gap open penalty (1; 2; 5; 10; 25; 50; 100), gap extension penalty (0.05; 0.2; 0.5; 1.0; 2.5; 5.0; 7.5; 10.0), gap distance penalty (1; 2; 3; 4; 5; 6; 7; 8; 9; 10), protein weight matrix (PAM developed by Dayhoff et al., 1978: Atlas of Protein Sequence and Structure, Dayhoff, MO, ed., NBRF, Washington:345; BLOSUM developed by Henikoff and Henikoff 1992: Proc. Natl. Acad. Sci. USA., 89:10915; GONNET updated matrix of Dayhoff et al., 1978: Atlas of Protein Sequence and Structure, Dayhoff, MO, ed., NBRF, Washington:345). When using ClustalW it is most preferred that the homologous polypeptide sequences and the inventive polypeptide sequence are clustered together by using the GONNET matrix in combination with gap open penalties higher than 5, gap extension penalties higher than or equal to 0.2, and gap distance penalties higher than 3. The number of homologous polypeptide sequences which may be clustered together with the inventive polypeptide sequence by the ClustalW alignment depends on the similarity of the analysed sequences. Analysis of polypeptide sequences with a high percentage of sequence identity will result in more homologous sequences being clustered together with the inventive sequence and analysis of polypeptide sequences with a lower

percentage of sequence identity will result in fewer homologous sequences being clustered together with the inventive sequence. It is most preferred that the polypeptide sequences for comparison included in the ClustalW, or PILEUP, or the like, multiple alignment have 60% identity or above with the inventive polypeptide sequence disclosed herein.

Eleven amino acid residues in the plant PEBP sequences have so far been identified as essential for a functional protein by crystallography (Banfield and Brady, 2000) or by mutation (Bradley et al., 1997; Ohshima et al., 1997; Pnueli et al., 1998). At these residues, LpTFL1 differs from the consensus at only one position (110) which is also the position with the highest degree of amino acid variation between species. It is postulated that the Serine residue at position 110 may confer the superior repressor activity of flowering demonstrated herein. Therefore, the polypeptides expressed from the polynucleotide fragments of the present invention may include the sequence of YESP(K/R) located between about residues 100 and 120, from the N-terminus.

The present invention further provides a method of significantly reducing or substantially preventing flowering in a perennial or biennial plant, the method comprising inserting an expression cassette into a plant host cell, the expression cassette comprising a promoter and a nucleotide sequence as shown in Figure 2, or a fragment or derivative thereof, growing the said transformed host cell in a suitable culture medium and expressing said DNA sequence to produce said protein, and wherein said expressed protein substantially reduces and/or prevents flowering in said plant. The promoter may be, for example, the monocot and dicot actin and ubiquitin promoters, monocot and dicot glyceraldehyde dehydrogenase (GAPDH) promoters, the cauliflower mosaic virus 35S (CaMV 35S) and 19S (CaMV 19S) promoters, the 35S CaMV promoter containing the translational enhancer (TMV omega element), the nopaline synthase (NOS) promoter, octopine synthase (OCS) promoter.

Expression of the flowering repressor protein may only be desirable for a limited period of time, for example, a limited number of seasons. Therefore, the polynucleotide of the present invention may be included in a controllable expression cassette wherein expression of the associated polynucleotide can be induced or reduced by an administered signal. In doing so, flowering will either become repressed or no longer be repressed and the plant will then proceed to flower.

In a yet further aspect the present invention provides a nucleotide sequence comprising a transcriptional regulatory sequence, a sequence under the transcriptional control thereof which encodes an RNA sequence characterised in that the RNA sequence is anti-sense to an mRNA which codes for LpTFL1 or functional homologues hereof.

The nucleotide sequence encoding the antisense RNA molecule can be of any length provided that the antisense RNA molecule transcribable therefrom is sufficiently long so as to be able to form a complex with a sense mRNA molecule encoding for LpTFL1. Thus, without the intention of being bound by theory it is thought that the antisense RNA molecule complexes with the mRNA for the protein or proteins and prevents or substantially inhibits the synthesis of a functional LpTFL1 or functional homologues hereof. As a consequence of the interference by the antisense RNA, flowering repressor activity of LpTFL1 or homologues hereof is substantially decreased or eliminated.

The DNA encoding the antisense RNA can be from about 20 nucleotides in length up to the length of the relevant mRNA produced by the cell. Preferably, the length of the DNA encoding the antisense RNA will be from 50 to 1500 nucleotides in length. The preferred source of antisense RNA transcribed from DNA constructs of the present invention is DNA showing substantial identity or similarity to LpTFL1.

Suppression of endogenous LpTFL1 expression can also be achieved using a ribozyme. Ribozymes are synthetic RNA molecules that possess highly specific endoribonuclease activity. The production and use of ribozymes are disclosed in U.S. Patent No. 4,987,071 to Cech and U.S. Patent No. 5,543,508 to Haselhoff. The inclusion of ribozyme sequences within antisense RNAs may be used to confer RNA cleaving activity on the antisense RNA, such that endogenous mRNA molecules that bind to the antisense RNA are cleaved, which in turn leads to an enhanced antisense inhibition of endogenous gene expression.

Vectors in which RNA encoded by the LpTFL1 cDNA (or variants thereof) is over-expressed may also be used to obtain co-suppression of the endogenous LpTFL1 gene in the manner described in U.S. Patent No. 5,231,020 to Jorgensen. Such co-suppression (also termed sense suppression) does not require that the entire LpTFL1 cDNA be introduced into the plant cells, nor does it require that the introduced sequence be exactly identical to the endogenous

LpTFL1 gene. However, as with antisense suppression, the suppressive efficiency will be enhanced as (1) the introduced sequence is lengthened and (2) the sequence similarity between the introduced sequence and the endogenous LpTFL1 gene is increased.

Vectors expressing an untranslatable form of the LpTFL1 mRNA may also be used to suppress the expression of endogenous LpTFL1 activity to induce flowering. Methods for producing such constructs are described in U.S. Patent No. 5,583,021 to Dougherty et al. Preferably, such constructs are made by introducing a premature stop codon into the LpTFL1 gene.

Alternatively, induction of flowering may be obtained by gene silencing using double-strand RNA (Sharp, 1999). This approach, whereby a vector is prepared in which a cDNA or gene is arranged in duplicated fashion and is capable of generating upon expression a double stranded RNA molecule with a hairpin structure. This procedure has been used to modify gene activity in plants (Chuang and Meyerowitz, 2000).

Another method for abolishing the expression of LpTFL1 is by insertion mutagenesis using the T-DNA of Agrobacterium tumefaciens. After generating the insertion mutants, the mutants can be screened to identify those containing the insertion in the LpTFL1 gene. Mutants containing a single mutation event at the LpTFL1 gene may be crossed to generate homozygous plants for the mutation (Koncz et al. (1992) Methods in Arabidopsis Research. World Scientific).

Flowering in a plant may also be controlled by recombinant systems such as the cre-lox system (for example, as described in US Pat. No. 5,658,772). A plant genome may be modified to include first and second lox sites that are then contacted with a Cre recombinase. If the lox sites are in the same orientation, the intervening DNA sequence between the two sites is excised. If the lox sites are in the opposite orientation, the intervening sequence is inverted.

The polynucleotides and polypeptides of this invention may also be expressed in a plant in the absence of an expression cassette by manipulating the activity or expression level of the endogenous gene by other means. For example, by ectopically expressing a gene by T-DNA activation tagging (Ichikawa et al., (1997) Nature 390 698-701, Kakimoto et al., (1996) Science 274: 982-985). This method entails transforming a plant with a gene tag containing multiple transcriptional enhancers and once the tag has inserted into the genome, expression of a flanking gene coding sequence becomes deregulated. In another example, the transcriptional machinery in

a plant may be modified so as to increase transcription levels of a polynucleotide of the invention (See PCT Publications W09606166 and WO 9853057 which describe the modification of the DNA binding specificity of zinc finger proteins by changing particular amino acids in the DNA binding motif).

The transgenic plant may also comprise the machinery necessary for expressing or altering the activity of a polypeptide encoded by an endogenous gene, for example by altering the phosphorylation state of the polypeptide to maintain it in an activated state.

Therefore, a further aspect of the present invention provides transgenic perennial or biennial plants wherein the flowering repressor activity attributable to LpTFL1 in the cells of the plants has been significantly reduced or substantially prevented. The plants may, for example, be monocots or dicots.

Transcriptional initiation sequences are commonly located upstream from the transcriptional initiation site and which contains all the regulatory regions required for transcription. Examples of such transcriptional initiation sequences (also known as promoters) are hereinbefore described.

It will be appreciated that the promoter employed should give rise to the transcription of a sufficient amount of the antisense RNA molecule at a rate sufficient to cause an inhibition of LpTFL1 activity in plant cells. The required amount of antisense RNA to be transcribed may vary from plant to plant.

DNA constructs and nucleotide sequences of the invention may be used to transform cells of both monocotyledonous and dicotyledonous plants in various ways known in the art. For example, particle bombardment of embryogenic callus is the method of choice for production of transgenic monocotyledonous plants [Vasil (1994) Plant Mol. Biol. 25, 925-937]. In many cases transformed plant cells may be cultured to regenerate whole plants which can subsequently reproduce to give successive generations of genetically modified plants.

The invention also provides a biological vector comprising a DNA construct according to the present invention. The biological vector may be a virus or bacterium, such as *Agrobacterium tumefaciens*, for example, and the construct advantageously further encodes a marker protein, such as one having herbicide resistance, or anti-bacterial properties.

A further aspect of the invention is a recombinant biological vector comprising the said construct wherein said vector is capable of transforming a host cell. Also comprised is a host cell stably transformed with the said vector wherein said host cell is preferably a cell selected from the group consisting of a bacterial cell, a yeast cell, and an insect cell and is further capable of expressing the polypeptide from the polynucleotide of the present invention.

The invention still further provides eukaryotic cells, such as plant cells (including protoplasts) for example, containing the said nucleotide sequence, DNA construct or vector.

The invention still further provides plant cell with gene "knockouts" wherein the gene encoding LpTFL1 or a functional fragment or derivative or homologue thereof has been mutated or removed to eliminate expression. Without wishing to be bound by theory, it is thought that this will result in early flowering by a plant.

The invention still further provides transgenic plants comprising such plant cells, the progeny of such plants which contain the sequence stably incorporated and heritable in a Mendelian manner, and/or the seeds of such plants or such progeny. Progeny of transgenic plants may be obtained by traditional vegetative propagation or by micropropagation.

The invention still further provides the use of the sequence according to the invention, whether "naked" or present in a DNA construct or biological vector, in the production of eukaryotic cells, particularly plant cells having a modified LpTFL1 activity.

Other plant LpTFL1 related coding sequences may be isolated according to well known techniques based on their sequence homology to the sequence as shown in Figure 2, or fragment, or homologue thereof. In these techniques all or part of the known LpTFL1 coding sequence may be used as a probe which selectively hybridises to other LpTFL1 coding sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e. genomic or cDNA libraries) from a chosen organism. Such techniques include hybridisation screening of plated DNA libraries (either plaques or colonies; see, e.g. Sambrook et al., "Molecular Cloning", eds., Cold Spring Harbor Laboratory Press. (1989)) and amplification by PCR using oligonucleotide primers, for example corresponding to sequence domains identified from the LpTFL1 gene sequence.

Therefore, a further embodiment of the invention is a method of isolating a polynucleotide fragment, said polynucleotide fragment comprising a sequence having at least 65% identity with

the sequence disclosed herein, such as 68%, 70%, 75%, 80%, 83%, 86%, 88%, 90%, 92%, 95%, 97% or 99% identity , said method comprising

(a) preparing a nucleotide probe capable of specifically hybridising to a plant LpTFL1 related gene or mRNA, wherein said probe comprises a contiguous portion of the coding sequence for LpTFL1 from ryegrass of at least 10 nucleotides in length;

(b) probing for other LpTFL1 related coding sequences in populations of genomic DNA fragments or cDNA fragments from a chosen plant using the nucleotide probe prepared according to step (a); and

(c) isolating a polynucleotide fragment comprising a portion encoding a protein having LpTFL1-like activity.

The isolated plant LpTFL1 and LpTFL1-related sequences taught by the present invention may be manipulated according to standard genetic engineering techniques to suit any desired purpose. For example, the entire LpTFL1 coding sequence or portions thereof may be used as probes capable of specifically hybridising to coding sequences and messenger RNAs. To achieve specific hybridisation under a variety of conditions, such probes include sequences that are unique among LpTFL1 coding sequences and are at least 10 nucleotides in length, preferably at least 20 nucleotides in length, and most preferably at least 50 nucleotides in length. Such probes may be used to amplify and/or analyse LpTFL1 coding sequences from a chosen organism via the well known process of polymerase chain reaction (PCR). This technique may be useful to isolate additional LpTFL1 related coding sequences from other plant species as hereinbefore described or as a screening assay to determine the presence of LpTFL1-related coding sequences in a plant. Hybridisation probes may also be used to quantitate levels of LpTFL1 mRNA in a plant using standard techniques such as Northern blot analysis.

LpTFL1-specific hybridisation probes may also be used, for example, to map the location of the native LpTFL1 related gene(s) in the genome of ryegrass using standard techniques based on the selective hybridisation of the probe to genomic LpTFL1 sequences. These techniques include, but are not limited to, identification of DNA polymorphisms identified or contained within the LpTFL1 probe sequence, and use of such polymorphisms to follow segregation of the LpTFL1 gene relative to other markers of known map position in a mapping population derived from self

fertilization of a hybrid of two polymorphic parental lines (see e.g. Helentjaris et al., Plant Mol. Biol. 5: 109 (1985); Sommer et al. Biotechniques 12:82 (1992); D'Ovidio et al., Plant Mol. Biol. 15: 169 (1990)). Mapping of the LpTFL1 gene in this manner is contemplated to be particularly useful for breeding purposes. For instance, by knowing the genetic map position of a mutant LpTFL1 related gene which could affect flowering, flanking DNA markers can be identified from a reference genetic map (see, e.g., Helentjaris, Trends Genet. 3: 217 (1987)). During introgression of the mutant LpTFL1 related gene trait into a new breeding line, these markers can then be used to monitor the extent of LpTFL1 related flanking chromosomal DNA still present in the recurrent parent after each round of back-crossing.

For recombinant production of the flowering repressor protein in a host organism, the *LpTFL1* DNA coding sequence may be inserted into an expression cassette to form a DNA construct designed for a chosen host and introduced into the host where it is recombinantly produced. The choice of specific regulatory sequences such as promoter, signal sequence, 5' and 3' untranslated sequences, enhancer and terminator appropriate for the chosen host is within the level of skill of the routine worker in the art. The resultant molecule, containing the individual elements linked in proper reading frame, may be introduced into the chosen cell, using techniques well known to those in the art, such as electroporation, biolistic introduction, Ti plasmid introduction etc. Suitable expression cassettes and vectors and methods for recombinant production of proteins are well known for host organisms such as E. coli (see, e.g. Studier and Moffatt, J. Mol. Biol. 189: 113 (1986); Brosius, DNA 8: 759 (1989)), yeast (see, e.g., Schneider and Guarente, Meth, Enzymol. 194: 373 (1991)) and insect cells (see, e.g., Luckow and Summers, Bio/Technol. 6: 47 (1988).

Examples of promoters suitable for use in DNA constructs of the present invention include viral, fungal, bacterial, animal and plant derived promoters capable of functioning in plant cells. The promoter may be selected from so-called constitutive promoters or inducible promoters.

Examples of suitable inducible or developmentally regulated promoters and constitutive promoters include the glutocorticoid-inducible transcription system, napin storage protein gene (induced during seed development), the malate synthase gene (induced during seedling germination), the small subunit RUBISCO gene (induced in photosynthetic tissue in response to

light), the patatin gene highly expressed in potato tubers, monocot and dicot actin and ubiquitin promoters, monocot and dicot glyceraldehyde dehyrogenase (GAPDH) promoters, the cauliflower mosaic virus 35S (CaMV 35S) and 19S (CaMV 19S) promoters, the 35S CaMV promoter containing the translational enhancer (TMV omega element), the nopaline synthase (NOS) promoter, octopine synthase (OCS) promoter, heat shock 80 (hsp 80) promoter, the maize Ubiqinine promoter, and the like. In addition, the inventors have identified a 3.6kb LpTFL1 promoter fragment upstream of the LpTFL1 DNA coding sequence, and this may be used as a promoter in an expression cassette. Therefore, in plants and plant cells of the invention, constitutive, inducible or developmentally regulated promoters are encompassed.

A terminator is contemplated as a DNA sequence at the end of a transcriptional unit which signals termination of transcription. These elements are 3'-non-translated sequences containing polyadenylation signals which act to cause the addition of polyadenylate sequences to the 3' end of primary transcripts. Sequences mentioned above may be isolated for example from fungi, bacteria, animals or plants.

Examples of terminators particularly suitable for use in the nucleotide sequence and DNA constructs of the invention include the nopaline synthase polyadenylation signal of Agrobacterium tumefaciens, the 35S polyadenylation signal of CaMV, octopine synthase polyadenylation signal, the zein polyadenylation signal from Zea mays, and those found in plasmids such as pBluescript (Stratagene, La Jolla, CA), pFLAG (International Biotechnologies, Inc., New Haven, CT), pTricHis (Invitrogen, La Jolla, CA), and baculovirus expression vectors, e.g., those derived from the genome of Autographica californica nuclear polyhedrosis virus (AcMNPV). An example baculovirus/insect system is pVl11392/Sf21 cells (Invitrogen, La Jolla, CA).

Therefore, the invention further provides an expression cassette comprising a promoter operably linked to a DNA coding sequence encoding LpTFL1, or a functionally active variant thereof and a terminator.

Recombinantly produced plant LpTFL1 protein or functionally active fragment can be isolated and purified using a variety of standard techniques. The actual techniques which may be used will vary depending upon the host organism used, whether the LpTFL1 protein or functionally active fragment is designed for secretion, and other such factors familiar to the skilled artisan (see,

e.g. chapter 16 of Ausubel, F. et al., "Current Protocols in Molecular Biology", pub. By John Wiley & Sons, Inc. (1994).

Therefore, the present invention further provides the recombinant production of LpTFL1, or a functionally active fragment thereof. In particular, the invention relates to a method of producing a protein having LpTFL1 activity in a host organism comprising

(a) inserting a DNA sequence encoding a protein having LpTFL1 activity into a host cell;

(b) growing the said transformed host cell in a suitable culture medium;

(c) expressing said DNA sequence to produce said protein; and

(d) isolating the protein product either from the transformed host cell or the culture medium or both and purifying it.

The cloning and expression of a recombinant LpTFL1 polypeptide fragment or functionally active fragment also facilitates in producing anti-LpTFL1 antibodies and fragments thereof (particularly monoclonal antibodies) and evaluation of in vitro and in vivo biological activity of recombinant a LpTFL1 polypeptide or functionally active fragment. The antibodies may be employed in diagnostic tests for a native LpTFL1 polypeptide.

Further aspects of the invention relate to methods and materials for reducing or preventing flowering in monocotyledonous plants. Thus, in one aspect, the present invention provides a method of significantly reducing or substantially preventing flowering in a monocotyledonous plant, the method comprising expressing a polypeptide from an isolated polynucleotide fragment comprising a nucleotide sequence as shown in Figure 2, or a fragment, derivative, or homologue thereof, in a monocotyledonous plant.

In a further aspect, the present invention provides a method of significantly reducing or substantially preventing flowering in a monocotyledonous plant, the method comprising expressing an isolated polypeptide having an amino acid sequence as shown in Figure 4, or a functionally active fragment, derivative or homologue thereof. The present invention also provides a method of significantly reducing or substantially preventing flowering in a monocotyledonous plant, the method comprising expressing a polypeptide from an isolated polynucleotide fragment comprising a similar nucleotide sequence, or fragments thereof, from other plants which are complementary to the one which hybridises under stringent or moderately stringent conditions with the nucleotide

sequences of the isolated polynucleotide fragments, or fragments thereof, disclosed herein. The invention still further provides a method of significantly reducing or substantially preventing flowering in a monocotyledonous plant, the method comprising expressing a polypeptide from an isolated polynucleotide fragment comprising a similar nucleotide sequence which is synthetic or artificial and is complementary to one which hybridises under stringent or moderately stringent conditions with the above disclosed nucleotide sequences, or fragments thereof. In addition, the present invention provides a method of reducing or substantially preventing flowering in a monocotyledonous plant, the method comprising expressing a polypeptide from an isolated polynucleotide fragment comprising the nucleotide sequence of bases selected from the group consisting of bases 1 to 912, bases 1 to 1624, bases -78 to 912, bases -78 to 1242, and bases -78 to 1624, all of Figure 3.

In another aspect, the present invention provides an isolated polynucleotide fragment having a nucleotide sequence of bases -3600 to -1 as shown in Figure 3, or a fragment or derivative thereof, for up-regulating gene expression in the apex and leaves of a monocotyledonous plant during conditions that lead to flowering The present invention further provides a method of significantly reducing or substantially preventing flowering in a monocotyledonous plant, the method comprising inserting an expression cassette into a plant host cell, the expression cassette comprising a promoter and a nucleotide sequence as shown in Figure 2, or a fragment or derivative thereof, growing the said transformed host cell in a suitable culture medium and expressing said DNA sequence to produce said protein, and wherein said expressed protein substantially reduces and/or prevents flowering in said plant.

In various further aspects, the present invention relates to materials and methods for reducing or preventing flowering in a plant. Thus, in one aspect, the present invention provides a method of significantly reducing or substantially preventing flowering in a plant, the method comprising expressing a polypeptide from an isolated polynucleotide fragment comprising a nucleotide sequence as shown in Figure 2, or a fragment, derivative, or homologue thereof, in a plant. The nucleotide sequence represents a gene sequence isolated from the ryegrass *Lolium perenne* which is hereinafter referred to as "LpTFL1".

In a further aspect, the present invention provides a method of significantly reducing or substantially preventing flowering in a plant, the method comprising expressing an isolated polypeptide having an amino acid sequence as shown in Figure 4, or a functionally active fragment, derivative or homologue thereof. The isolated polypeptide is encoded by the isolated polynucleotide fragment as shown in Figure 2, or a fragment, derivative, or homologue thereof.

The present invention also provides a method of significantly reducing or substantially preventing flowering in a plant, the method comprising expressing a polypeptide from an isolated polynucleotide fragment comprising a similar nucleotide sequence, or fragments thereof, from other plants which are complementary to the one which hybridises under stringent or moderately stringent conditions with the nucleotide sequences of the isolated polynucleotide fragments, or fragments thereof, disclosed herein. The isolated polynucleotide fragments comprising a similar nucleotide sequence employed in the present invention may be isolated from any plant including monocots and dicots and in particular agriculturally important plant species, including but not limited to, crops such as those belonging to the grass family of *Poaceae*, and also soybean, potato, oilseed rape, sunflower, alfalfa, sugar cane and cotton; or herbs such as anise, basil, bay laurel, caper, caraway, cayenne pepper, celery, chervil, chives, coriander, cumin, dill, fennel, garlic, horseradish, leeks, lemon balm, liquorice, marjoram, mint, oregano, parsley, rosemary, sesame, tarragon and thyme; or fruits and vegetables, such as banana, blackberry, blueberry, strawberry, and raspberry, cantaloupe, carrot, cauliflower, coffee, cucumber, eggplant, grapes, honeydew, lettuce, mango, melon, onion, papaya, peas, peppers, pineapple, spinach, squash, sweet corn, tobacco, tomato, watermelon, rosaceous fruits (such as apple, peach, pear, cherry and plum) and vegetable brassicas (such as broccoli, cabbage, cauliflower, brussel sprouts, beet and kohlrabi). The homologues may also be derived from woody species, such as eucalyptus, oak, pine and poplar. The plant may further be an annual, a biennial or a perennial.

The invention also provides a method of significantly reducing or substantially preventing flowering in a plant, the method comprising expressing a polypeptide from an isolated polynucleotide fragment comprising a similar nucleotide sequence which is synthetic or artificial and is complementary to one which hybridises under stringent or moderately stringent conditions with the above disclosed nucleotide sequences, or fragments thereof.

In addition, the present invention provides a method of reducing or substantially preventing flowering in a plant, the method comprising expressing a polypeptide from an isolated polynucleotide fragment comprising the nucleotide sequence of bases selected from the group consisting of bases 1 to 912, bases 1 to 1624, bases -78 to 912, bases -78 to 1242, and bases -78 to 1624, all of Figure 3.

In another aspect, the present invention provides an isolated polynucleotide fragment having a nucleotide sequence of bases -3600 to -1 as shown in Figure 3, or a fragment or derivative thereof, for up-regulating gene expression in the apex and leaves of a plant during conditions that lead to flowering.

The present invention further provides a method of significantly reducing or substantially preventing flowering in a plant, the method comprising inserting an expression cassette into a plant host cell, the expression cassette comprising a promoter and a nucleotide sequence as shown in Figure 2, or a fragment or derivative thereof, growing the said transformed host cell in a suitable culture medium and expressing said DNA sequence to produce said protein, and wherein said expressed protein substantially reduces and/or prevents flowering in said plant. The promoter may be, for example, the monocot and dicot actin and ubiquitin promoters, monocot and dicot glyceraldehyde dehyrogenase (GAPDH) promoters, the cauliflower mosaic virus 35S (CaMV 35S) and 19S (CaMV 19S) promoters, the 35S CaMV promoter containing the translational enhancer (TMV omega element), the nopaline synthase (NOS) promoter, octopine synthase (OCS) promoter.

Expression of the flowering repressor protein may only be desirable for a limited period of time, for example, a limited number of seasons. Therefore, the polynucleotide of the present invention may be included in a controllable expression cassette wherein expression of the associated polynucleotide can be induced or reduced by an administered signal. In doing so, flowering will either become repressed or no longer be repressed and the plant will then proceed to flower.

A further aspect of the present invention provides transgenic plants wherein the flowering repressor activity attributable to LpTFL1 in the cells of the plants has been significantly reduced or substantially prevented. The plants may, for example, be monocots or dicots, and may further be annuals, biennials or perennials.

DNA constructs and nucleotide sequences of the invention may be used to transform cells of both monocotyledonous and dicotyledonous plants in various ways known in the art. For example, particle bombardment and Agro-mediated transformation of embryogenic callus is the method of choice for production of transgenic monocotyledonous plants [Vasil (1994); Tingay *et al.* (1997); Hiei *et al.* (1994)]. In many cases transformed plant cells may be cultured to regenerate whole plants which can subsequently reproduce to give successive generations of genetically modified plants.

Detailed Description of the Invention

These and other aspects of the present invention will now be described by way of example only, in conjunction with the accompanying Figures, in which:

Figure 1 illustrates the comparative morphology of perennial ryegrass and Arabidopsis. (A) The ryegrass vegetative apex is very compact with the SAM and the semicircular ridges that later will give rise to leaves and tillers. It is positioned on the basal crown and surrounded by developing leaves. Bar = 1.0mm. (B) The ryegrass inflorescence consists of spikelets alternately attached to the main axis (rachis). Each spikelet consists of three to 10 flowers. Bar = 1.0mm. (C) Schematic diagrams of ryegrass and Arabidopsis. During vegetative growth the SAM-of ryegrass and Arabidopsis produce very closely spaced leaves in a rosette. After the floral transition the SAM of both species elongate (bolt) and floral organs (circles) are produced along the main axis. In both plants secondary shoots arise from the axils of subtending leaves. In Arabidopsis wild type, flowers mature in an acropetal order and the SAM grows indefinitely (arrowheads), whereas in the *tfl1* mutant the SAM and the secondary shoots terminate in a flower. Like the *tfl1* mutant, the ryegrass SAM and secondary shoots also terminate in a flower. Maturation of flowers in the ryegrass inflorescence is basipetal, and all the secondary shoots formed below the apex also develop into arrays of flowers in a cymose pattern. The collar is a special meristematic region on the leaf blade in the junction between the leaf blade and the stem (black circles). An enlargement of a floret is shown (redrawn from K. Esau, Anatomy of Seed Plants, Ed 2. Wiley and Sons, New York, 1977). Each floret consists of four whorls of organs. The outermost whorl consists of the palea and the

lemma surrounding the lodicules (whorl 2), the three stamens (whorl 3), and the ovary (whorl 4), which is interpreted as syncarpous, consisting of two or three carpels forming the ovary (C);

Figure 2 illustrates the cDNA sequence of the LpTFL1 gene;

Figure 3 illustrates the genomic sequence of the LpTFL1 gene (bases -78 to 1624) and the upstream promoter region (bases -3600 to -1);

Figure 4 is the polypeptide sequence derived from the polynucleotide sequence of Figure 2;

Figure 5 illustrates the genomic organisation of *LpTFL1* and similarity of the deduced protein with other plant PEBPs. (A) The upper bar shows the genomic organisation of the gene, including the untranslated (black boxes) and the translated (white boxes) regions. A 180bp DNA fragment was isolated from ryegrass by RT-PCR. (B) Comparison of the deduced protein sequence for the *LpTFL1* gene (accession no. AF316419) with those of *TFL1* (Bradley et al., 1997; Ohshima et al., 1997), *CEN* (Bradley et al., 1996), *SP* (Pnueli et al., 1998), *BNTFL1-1* and *BNTFL1-3* (Mimida et al., 1999), *CET1, CET2,* and *CET4* (Amaya et al., 1999), *FDR1* and *FDR2* (accession nos. AAD42896 and AAD42895, respectively), and *FT* (Kardailsky et al., 1999; Kobayashi et al., 1999). CLUSTAL W program was used to make the alignment and the deduced distance tree. Identical residues are in black. Dashed lines indicate gaps introduced by the program to achieve maximum alignment. Identical intron positions among all species are marked with black arrowheads. White arrowheads indicate amino acids identified to be at the ligand-binding sites by crystallography (Banfield and Brady, 2000) and asterisks indicate amino acids in which point mutations were described for Arabidopsis (Bradley et al., 1997; Ohshima et al., 1997) and tomato (Pnueli et al., 1998). (C) Distance tree of different plant PEBPs. The lengths of the horizontal lines are proportional to the similarity between the predicted protein sequences;

Figure 6 illustrates the LpTFL1 mRNA levels in various tissues detected by Real-time quantitative RT-PCR Five micrograms of RNA from each of eleven different tissue samples which included flowers, stems, roots, leaves from non-induced (control), 12 weeks vernalized and five weeks long day (LD) induced plants and apices from control, 6 and 12 weeks vernalized and 5 weeks LD induced plants was used for the analysis. Levels of the constitutively expressed GAPDH

mRNA were measured simultaneously, and the amount of LpTFL1 mRNA in each sample was normalised to the amount of GAPDH.

Figure 7 illustrates that UBI-*LpTFL1* dramatically alters the duration of the vegetative phase of Arabidopsis. (A) RNA gel-blot analysis of primary transformants (lines 1-30) and wild-type plants (WT). Fifteen micrograms of RNA from rosette leaves was blotted and probed with a *LpTFL1* cDNA probe. Transgenic lines 5, 16, 29, and 30 have single-copy insertions as detected by DNA-blot analysis (not shown). Lines 2, 7, 9, 11, and 13 were non-flowering. (B) Expression of *AP1* and *ACTIN* in a *tfl1-14* mutant line, wild type, and in a UBI::*LpTFL1* plant (line 17) as detected by RT-PCR on 5µg of RNA from each plant. C, Number of cauline leaves produced on the main stem in *tfl1* mutant, the complemented mutant *(tfl1*)*, wild type (WT), and UBI-*LpTFL1* primary transformants (groups A-D). Each bar represents the mean value of the plants within the specific group. Numbers above the bar indicates the total number of days from germination till the onset of the first flower. The plants were grouped according to the time to flowering: (A ≥ 75 d; B ≥ 100 d; C ≥ 150 d; D non-flowering [NF]) The number of plants in each group is *tfl1*, 6; *tfl1**, 6; WT, 6; A, 6; B, 13; C, 5; and D, 5.

Figure 8 illustrates the effect of UBI-*LpTFL1* on the morphology of Arabidopsis. (A) The UBI::*LpTFL1* Arabidopsis primary transformants, line 1 and 2 (right-hand side), showing extensive vegetative growth and up to fourth-order branching 4 months after germination compared with a 1-month-old flowering wild-type plant (left side). Line 2 (middle) was non-flowering after 7 months of growth. (B) The SAM of most UBI::*LpTFL1* Arabidopsis lines is compact, filled with leaf primordia, and covered with trichomes. (C) and (D) Trichome distribution on the adaxial surface of the uppermost cauline leaves on the main stem of UBI::*LpTFL1* (C) compared with wild-type cauline leaves at same age (D). (E) In the *UBI::LpTFL1* plants leafy shoots filled with trichomes are produced in place of normal flowers on the upper coflorescences.

Figure 9 illustrates the integration of LpTFL1 transgene by PCR analysis of genomic DNA from non-transformed (CON) and basta-resistent ryegrass lines (numbers). Primer LP0 was used in combination with primer MS8 to amplify a 560-bp fragment. Approximately 0.3 µg of genomic DNA was used per reaction.

Figure 10 illustrates the number of inflorescences produced by the non-transformed (CON) and the transgenic UBI::*LpTFL1* ryegrass lines, compared with the relative levels of *LpTFL1* mRNA (black bars, second Y-axis). All *LpTFL1* mRNA levels are relative to the level of *LpACTIN*, and the highest detected value was set to 100 (line 36). Horizontal bars below the graph indicates, which of the lines were tested positive for stable integration of *LpTFL1* into the genome.

Figure 11 illustrates the DNA blot hybridisation analyses of genomic DNA from non-transformed (CON 1) and transgenic red fescue. DNA samples of 10-30 µg were restricted with *Hin*DIII (A) or *Eco*RI (B) and probed with a 0.4-kb fragment containing the 3' -end of the ubiquitin intron and the 5' -end of the *LpTFL1* coding region (MS56-LP4REV, see Table 1). *Hin*DIII release from pLPTFL1 a 2.8-kb fragment containing the entire *LpTFL1* cassette (arrowhead). *Eco*R1 has a single restriction site on pLPTFL1, which is a *5.5* -kb plasmid (arrowhead);

Figure 12 illustrates the transgene levels and phenotypes of the transgenic UBI::*LpTFL1* fescue lines. A. Average number of spikes produced per clone during the first (grey bars) and the second (checked bars) season by the lines (A-N), the non-transformed (CON), and the transformation controls (BAR), compared with the relative levels of *LpTFL1* mRNA (black bars, second Y-axis). The white bar represents the level of a transcript corresponding to a truncated *LpTFL1* mRNA. B. The average stem length of each line measured during the first season (grey bars) and compared with *LpTFL1* mRNA levels (black bars, second Y-axis). Error-bars show the standard deviation from the average value within each line. All *LpTFL1* mRNA levels are relative to the level of *LpACTIN*, and the highest detected value was set to 100 (line J).

Figure 13 illustrates an RNA gel blot analysis of primary transformants from different UBI::*LpTFL1* lines. 2.5µg of poly-$A^+$ mRNA each line were blotted and probed with a 200 bp *LpTFL1* or a 450 bp LpAC*TIN* cDNA probe. All the lines included were positive for the transgene as was verified by PCR (lower panel) using the primers MS56 and Lp4REV which amplifies a 425 bp fragment corresponding to the last 75 bases in the ubiquitin 3' end and 350 bases of the *LpTFL1* cDNA;

Figure 14 illustrates the flowering and non-flowering plants of 400 days old fescue wild-type (CON), transgenic controls (BAR) and UBI::*LpTFL1* lines (A-M). Each line represents a single transformation event. Black bars below each picture shows to the *LpTFL1* transcript level relative to

the level in the non-transformed control plants, which was set to 1.0. The white bar indicates that the level corresponds to level of an overexpressed truncated *LpTFL1* transcript; and

Figure 15 illustrates the Panicle phenotypes of red fescue wild-type (CON1) and of the UBI::*LpTFL1* transgenic lines A and C, which overexpress a truncated and a correct *LpTFL1* transcript, respectively. Bar = 1 cm.

Figure 16 is a table showing the transformation efficiency and the floral activity for a number of transgenic ryegrass lines transformed with *LpTFL1*.

Figure 17 shows analysis of transgene integration by PCR in UBI::*LpTFL1* transgenic red fescue lines.

**EXAMPLES SECTION**

**1. EXPRESSION OF LpTFL1 IN ARABIDOPSIS**

**MATERIALS AND METHODS**

***Plant Growth Conditions***

Ryegrass *(Lolium perenne)* plants (clone F6, DLF-TRIFOLIUM) were grown in soil in a greenhouse with daylight at 21 and 18°C, day and night temperature, respectively. For the primary induction (vernalization) plants were grown in a growth chamber at or below 5°C for at least 12 weeks. During vernalization, the light period was decreased to 8 h per day. Following vernalization, plants were grown under 16 h of light at 22°C and 18°C, day and night temperature, respectively, for secondary induction. For RNA analysis wild-type plants were harvested before vernalization, after 6 weeks of vernalization, and after 14 and 28 d of secondary induction and meristems were excised. Samples from other tissues like leaves, stems, seeds, and roots were also harvested for expression analysis.

Arabidopsis seeds were stratified for 2 to 3 d at 4°C and then grown in soil in growth chambers at 22°C and 18°C, day and night temperature, respectively. During the first 2 weeks plants were grown at SD conditions (8 h of light per day) and then moved to LD conditions (16 h of light per day). In the Arabidopsis time-course experiment rosette leaves were counted when plants started to bolt and the number of leaf nodes were counted from the most basal cauline leaf to the

uppermost leaf proximal to the inflorescence. The number of days from germination to the production of the first flower-like structure was also scored.

***Screening of cDNA and Genomic Library***

To isolate plant PEBP genes from ryegrass, a set of primers partially homologous to *TFL1* of Arabidopsis, *CEN* of *Antirrhinum,* and a rice EST (RICR2918A; accession no. 428842) were designed. Primer RY2 N (5'-GGTTATGACAGACCCAGATGTG-3') was used in combination with primer RY4V (5'-CGAACCTGTGGATACCAATG-3') to amplify a 180-bp fragment by RT-PCR. Preparation of RNA for the RT-PCR used the FastRNA, GREEN Kit RNA isolation system (Bio101, Carlsbad, CA). The 180-bp fragment was used to screen a cDNA library (Stratagene, La Jolla, CA) made of ryegrass inflorescences for full-length cDNAs. Approximately 800,000 recombinants were screened at moderate stringency of 60°C , with washes at 60°C in 2x SSC (0.3M NaCl and 0.1M sodium citrate, pH 7.4) and 0.1% (w/v) SDS. Three positive clones were isolated, and plasmids were isolated from single plaques by in vivo excision. All cDNA clones were sequenced and contained identical sequences with similarity to *TFL1* and *CEN* and were named ryegrass *TFL1*-like, *LpTFL1* (GenBank accession no. AF316419).

A λEMBL3 5P6/T7 genomic library (CLONTECH, Palo Alto, CA) made from a partial *Sau3A* digest of ryegrass DNA was screened for *TFL1*-like genes. Approximately 1,000,000 recombinants were screened at moderate stringency (as described above) with the full-length *LpTFL1* cDNA clone. Nine positive clones were isolated and digestion of the λ DNA clones with *Bam*H1, *Sal*I, *Xba*I, and *Sac*I revealed three unique clones. These clones were partially sequenced and all three had identical sequence from 4.0 kb upstream and 2.0 kb downstream of the *LpTFL1* sequence. The sequence of the exons of the genomic clones, as well as the 5' and 3' untranslated region were identical to *LpTFL1.* DNA sequencing was performed using the ABI Prism system (Perkin-Elmer, Foster City, CA), and sequence analysis and alignments were produced using Gene Codes Sequencer software, version 4.02.

***RNA/DNA Analysis***

For detection of *LpTFL1* mRNA level in different organs of ryegrass at different stages poly-$(A)^+$ mRNA was isolated from 5 µg of total RNA from each tissue sample and all mRNA was used in the reverse transcription. Two internal primers, INS5 (5'-CACATTGGTTATGACGGACC-3') and INS3 (5'-CTCCCCCCCAAATGAAGC-3'), were used in the subsequent Real-time quantitative PCR reaction to amplify a 200-bp *LpTFL1* fragment from the first strand cDNA templates. Amplification of PCR products were performed and monitored by the LightCycler™ system in which inclusion of the fluorescent dye SYBR green I Dye into the reaction mixture facilitates direct measurement of double stranded DNA after each PCR cycle. In this experiment, the *LpTFL1* expression level was analyzed in eleven different tissue sample which included flowers, stems, knees, roots, leaves from non-induced (control), 12 wk vernalized and 5 wk long day (LD) induced plants and apices from control, 6 and 12 wk vernalized and 5 wk LD induced plants. One microlitre of each RT-reaction were used as template in the PCR reaction together with dilutions of plasmids containing *LpTFL1* and *LpGAPDH* (100, 10, 1 and 0.1 pg). In order to normalize the PCR results relative to the initial template amount in each sample, a PCR was run in parallel on similar samples with the primers GAP5 (5'-CAAGGACTGGAGAGGTGG-3') and GAP3 (5'-TTGACTCGTTGTCGTACC-3') to amplify a 380 bp *LpGAPDH* fragment. Detection of *LpTFL1* RNA levels in transformed Arabidopsis was performed by standard RNA gel-blot analysis.

***Construction of UBI::LpTFL1 and the Transformation of Arabidopsis Wild Type and tfl1 Mutants***

The coding region of *LpTFL1* cDNA was amplified using primers B0 (5'-GGATCCCCATGTCTAGGTCTGTGGAG-3') and B550 (5'-GGGATCCCACAACTGGGATAG-CCA-3') and recombinant *pfu* polymerase. The fragment was blunt ligated into vector pAHC27 (Christensen and Quail, 1996) containing the maize *Ubiquitin* promoter, an exon:intron region, and the NOS terminator. The entire cassette (UBI::EXintron::*LpTFL1*::NOS) was excised from the plasmid by digestion with *Hind*II and *Eco*RI and was ligated into the *Eco*RI-*Hind*II site of the binary vector pCAMBIA3300 (Jefferson, Australia), which confers BASTA resistance, to give pCAMLPTFL1. Arabidopsis plants (Columbia and *tfl1-14* mutants) were transformed with

*Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986) harbouring the pCAMLPTFL1 (for *LpTFL1* overexpression) using the floral dip method described by Clough and Bent (1998).

## RESULTS

### *Isolation of a* plant PEBP *Gene from Ryegrass*

LpTFL1 was amplified from ryegrass inflorescence mRNA by reverse transcriptase (RT) PCR using primers designed on the basis of an alignment of *Arabidopsis TFL1*, *Antirrhinum CEN,* tomato *SP,* and a related rice expressed sequence tag (EST; Fig. 5A). This fragment was used to screen a ryegrass flower cDNA library at moderate stringency for plant PEBP genes *(LpTFL1* genes). One full-length cDNA was identified. The coding region of this cDNA shows 87% and 85% DNA sequence identity to two rice genes, *FDR2* and *FDR1,* and 67% and 64% identity with *TFL1* and *CEN,* respectively. The region in the rice EST used to design the *LpTFL1*-specific primers is 86% identical to *LpTFL1.* On the protein level LpTFL1 shows 91% and 86% identity to the corresponding proteins, FDR2 and FDR1, respectively, and 71% and 68% identity to TFL1 and CEN (Fig. 5B). The *LpTFL1* cDNA-coding region shows 60% identity with the *FT* sequence, and the protein identity is 56%. Comparison of LpTFL1 sequence with other plant PEBP sequences found in the database revealed that LpTFL1 groups together with the two rice proteins and also CET1 from tobacco (Fig. 5C). Banfield and Brady (2000) have recently determined the three-dimensional structure of the *CEN* protein and identified the amino acids essential for a functional ligand-binding site. Other amino acids important for a functional protein have been identified by mutation (Bradley et al., 1997; Ohshima et al., 1997; Pnueli et al., 1998) (Fig. 5B). Of these 11 functionally important amino acids, LpTFL1 differs from CEN at only one position (110), having a Ser instead of a Met.

A DNA-blot analysis at moderate stringency using a full-length *LpTFL1* cDNA fragment as probe was performed to assess the number of Plant PEBP genes in ryegrass. The results indicate that two Plant PEBP genes are present in ryegrass (data not shown). To gain more information on the *LpTFL1* gene we screened a ryegrass genomic library with the full-length *LpTFL1* cDNA clone. Three independent genomic clones were retrieved and sequenced. All had an identical DNA sequence predicting the same open reading frames, which exactly matched the *LpTFL1* cDNA.

The *LpTFL1* genomic sequence contains three introns of 100, 208, and 82 bp , respectively.. In the approximately 3.6kb region upstream of the transcription start, no likely gene encoding open reading frames were found; this is the *LpTFL1* promoter.

***The LpTFL1 Gene in Ryegrass Shows an Expression Pattern Different from TFL1 in Arabidopsis***

To determine the expression pattern of *LpTFL1* message in ryegrass we examined the mRNA levels in different tissues by the LightCycler™ real time quantitative PCR. In order to compare the different samples in the PCR, the calculated *LpTFL1* template concentrations had to be normalized to the levels of *LpGAPDH* template concentration (Table 1). We assumed that *LpGAPDH* is constitutively expressed in all cells at different time points.

*LpTFL1* message was detectable in all types of tissue tested (Fig. 6). In the vegetative ryegrass, LpTFL1 mRNA was expressed at very low levels both in the apex as well as in the leaves. During vernalization the expression of LpTFL1 mRNA was slightly upregulated in the apex but remains low in the leaves. However during the subsequent LD treatment the level of LpTFL1 mRNA expression was significantly increased both in the apex (12.6 fold) and even more in the leaves (27.8 fold). Highest expression was observed in roots. The LD induced upregulation of *LpTFL1* expression in ryegrass has not been reported for any other plant PEBP so far identified, and expression of plant PEBPs outside the meristematic regions have only been detected in tobacco and tobacco by RT-PCR (Bradley et al., 1996; Amaya et al., 1999).

***LpTFL1 Delays or Prevents Flowering in Arabidopsis***

*LpTFL1,* one of the two Plant PEBP genes in ryegrass, has a function similar but noticeably stronger than the Arabidopsis TFL1. We used the maize ubiquitin promoter (Christensen and Quail, 1996) to drive overexpression of the *LpTFL1*-coding region in Arabidopsis. Following transformation with UBI::*LpTFL1,* 33 BASTA-resistant Arabidopsis plants were obtained. All the transformants showed remarkable vegetative characteristics and were much delayed in flowering compared with the wild type (Figs. 4 and 5). Whereas wild-type plants bolted 10 d after they were moved from SD (short day) to LD photoperiod, even the earliest flowering UBI::*LpTFL1* plants

required another month in LD before they bolted. After 3 months, more than one-half of the plants had not produced a single flower (Fig. 7C). Overexpression of *LpTFL1* affected the vegetative and the early inflorescence stage of Arabidopsis, as observed by the increased number of nodes produced before and after bolting. During the vegetative phase, wild-type Arabidopsis plants produced 16 ± 1.9 rosette leaves, whereas the UBI::*LpTFL1* plants grown under the same conditions produced 33.9 ± 8.9 rosette leaves (not shown). After the plants had bolted, the UBI::*LpTFL1* plants produced 26 ± 14.3 cauline leaves on the main stem before flowering, in contrast to the wild type, which produced only 4.8 ± 0.4 cauline leaves (Fig. 7C). Thus, in terms of time and the number of nodes produced before flowering, the majority of the UBI::*LpTFL1* plants appeared to be arrested in the early inflorescence phase. Overexpression of *TFL1* in *Arabidopsis* driven by the 35S cauliflower mosaic virus (CaMV) promoter has also been described (Ratcliffe et al., 1998). However, the 35S::*TFL1* plants produced only two-thirds of the number of rosette leaves and one-half of the number of cauline leaves compared with the UBI::*LpTFL1* plants, when grown under continuous light (Ratcliffe et al., 1998). Five *UBI::LpTFL1* plants (lines 2, 7, 9, 11, and 13) remained in the early inflorescence stage throughout their life cycle and failed to produce flowers before they senescenced and died (after 7 months). This is an extraordinary observation since non-flowering individuals has not been observed in 35S::*TFL1* Arabidopsis grown under LD inductive conditions. Only when 35S::*TFL1* Arabidopsis plants were grown under SD non-inductive conditions a few lines remained without flowers (Ratcliffe et al., 1998).

In addition to the main SAM, Arabidopsis plants transformed with UBI::*LpTFL1* also exhibit abnormal axillary meristem development. The development of coflorescences with developing flowers in the axils of the cauline leaves normally observed in wild-type Arabidopsis was rarely seen in the UBI::*LpTFL1* plants. However, in the place of floral organ formation, a "leafy" branch was produced, resulting in a highly branched, bushy, and dramatic phenotype (Fig. 8A). Third-order branching was a common trait among the UBI::*LpTFL1* plants, and fourth-order branching was observed in a single plant (Fig. 8A, right-hand plant). A reiterative series of leaves was continuously produced from the SAM of the UBI::*LpTFL1* plants, most of them with a high density of trichomes (Fig. 8B). The trichome distribution on the surface of the cauline leaves was in general much more dense than in the wild type (Fig. 8C and 8D). Increased trichome production in relation

to *TFL1* overexpression in Arabidopsis has not previously been reported. The disappearance of adaxial trichomes is a marker for loss of juvenility (Chien and Sussex, 1996; Telfer et al., 1997) and the continuation of trichome production therefore must reflect the vegetative nature of the UBI::*LpTFL1* plants. Compared with the wild type, most of the UBI::*LpTFL1* plants produced remarkably more and longer internodes on the main stem, and also on the coflorescences. In contrast to the wild-type plants, the uppermost coflorescences without the subtending cauline leaf of the UBI::*LpTFL1* plants did not consist of normal solitary flowers, but instead a leaf-like shoot (Fig. 8E).

Based on the time to flowering, the transformants could be grouped into four classes (A-D) displaying a phenotype from late flowering (Fig. 7C, group A) to -never flowering (Fig. 7C, group D). RNA gel-blot analysis revealed that most of the UBI::*LpTFL1* plants showed strong expression of *LpTFL1* (Fig. 7A, lines 1-31). Overall, the severity of the UBI::*LpTFL1* plant phenotypes was positively correlated with the level of *LpTFL1* expression in the corresponding plants, and the levels of *LpTFL1* expression in the non-flowering lines 2, 7, 9, 11 and 13 were among the highest of all lines tested, The expression level of *LpTFL1,* in turn, was positively correlated with the total number of gene copies inserted in the genome, as determined by DNA gel-blot analysis (data not shown). In plants with a single-copy insertion (Fig. 7A, lines 5, 16, and 29-30), the *LpTFL1* RNA levels were reduced compared with other lines and consequently the phenotype was less severe, but the time to flowering was still significantly longer than in the wild type (Fig. 7C, group A). Three BASTA-resistant plants in which *LpTFL1* expression was not detected by gel-blot analysis looked similar to wild-type plants with respect to their morphology, but flowered 10 d later than the wild type (not shown).

***LpTFL1 Overexpression in a tfl1-14 Mutant Background***

To further address the functional properties of LpTFL1 we asked if LpTFL1 is able to complement the Arabidopsis tfl1-14 strong mutant allele. In this mutant a C to T mutation leads to a Thr to iso-Leu substitution at position 69 (Fig. 5B). The *tfl1-14* mutant has a short vegetative phase and exhibits reduced plant height with few nodes, increased number of inflorescence arising from the rosette axillary meristems, and a determinate growth pattern (Bradley et al., 1997; Ohshima et

al., 1997). The construct used for transformation of the Arabidopsis wild type was also used for transformation of the *tfl1-14* mutant. More than 100 independent UBI::*LpTFL1-tfl1-14* primary transformants were obtained from each mutant line after selection for the binary plasmid. All the plants displayed a variety of phenotypes from wild type to the same extended vegetative phenotype seen in the UBI::*LpTFL1* wild-type background. On average (taken only from the first six plants flowering) the UBI::*LpTFL1-tfl1-14* plants produced 15.2 ± 3.5 cauline leaves on the main stem and flowered 33 d later than the *tfl1-14* mutant and 23 d later than the wild type (Fig. 7C). All the UBI::*LpTFL1-tfl1-14* plants grew indefinitely and the production of terminal flowers and rosette inflorescence, which is always seen in the *tfl1-14* mutants, was never observed in the transformants. Thus, the *LpTFL1* rescued the Arabidopsis tfl1-14 mutant in terms of morphology, and further extended the vegetative appearance.

**DISCUSSION**

Perennial ryegrass is a forage grass with a high agronomic value, since it is a low-cost crop, it is perennial, and it is widely used for feeding cattle. One of the major goals in crop improvement is the control of reproductive growth and flower development. Molecular information on these events is very limited in this species. We have isolated a Plant PEBP gene from perennial ryegrass, which is shown to be a repressor of flowering and involved in control of axillary meristem identity.

***Lolium LpTFL1 Is a New Member of the Plant PEBP Family***

The ryegrass Plant PEBP gene, *LpTFL1,* encodes a protein with high homology to a group of plant proteins that share structural similarities to mammalian PEBPs. Based on these similarities the plant PEBPs are predicted to play a role in the regulation of signalling cascades as has been shown for the mammalian PEBPs (Yeung et al., 1999; Banfield and Brady, 2000). The two proteins most similar to LpTFL1 are the rice FDR2 and FDR1 with 91% and 86% identity, respectively. In a multiple comparison including Plant PEBP proteins from different species, as well as FT from Arabidopsis, LpTFL1 is grouped together with the two rice proteins and a tobacco CEN-like protein,

CET1. No data on *FDR2/FDR1* expression patterns and functions in rice has been reported, and for *CET1*, expression has been reported to be detectable in vegetative and inflorescence shoots, but only by RT-PCR (Amaya et al., 1999). Compared with the Arabidopsis PEBP sequences, LpTFL1 shows 71% identity to TFL1 and 56% identity to FT. FT, which is 56% identical to the TFL1 protein, also belongs to the family of plant PEBPs, however, in contrast to TFL1, FT has been shown to mediate flowering-inducing signals in Arabidopsis (Kardailsky et al., 1999; Kobayashi et al., 1999). In this process FT acts in parallel with and under the influence of the *CONSTANS (CO)* gene, which is a mediator of the LD-induction pathway (Samach et al., 2000). LpTFL1 shows 50% identity to a partial FT-like region on a rice clone (nbxb0035E07r), but although the DNA-blot analysis indicates the existence of another *LpTFL1*-like gene, no ryegrass FT-like cDNA with a higher homology to this partial rice FT-like sequence has yet been identified. Overexpression of *LpTFL1* in Arabidopsis results in significantly delayed flowering in combination with a dramatic large and bushy phenotype, suggesting that LpTFL1 is more TFL1-like than FT-like.

In spite of the high degree of homology between the plant PEBPs, constitutive expression of these proteins in different plants leads to different phenotypes. The dramatic impact of *LpTFL1* overexpression on floral transition and plant architecture in Arabidopsis is more extreme than that previously reported by overexpressing *TFL1* in Arabidopsis (Ratcliffe et al., 1998). It could be speculated that the more severe phenotype observed in our study may be that the activity of the maize ubiquitin promoter is stronger than the 35S CaMV promoter in Arabidopsis. However, this would require the monocot ubiquitin promoter having a remarkably strong activity not previously reported in a dicot plant. We suggest that our observation may be due to differences in the protein sequence and conformation of LpTFL1 compared with TFL1. Overexpression of *CEN* in tobacco has also been reported to significantly delay the floral transition, as well as to change the plant architecture (Amaya et al., 1999). In contrast, there was no effect of overexpressing *TFL1* in tobacco (Amaya et al., 1999). These results, together with our results, indicate that differences in the protein sequences among the plant PEBPs are likely to account for the differences observed in the overexpressing plants.

Eleven amino acid residues in the plant PEBP sequences have so far been identified as essential for a functional protein (Fig. 5B) by crystallography (Banfield and Brady, 2000) or by

mutations (Bradley et al., 1997; Ohshima et al., 1997; Pnueli et al., 1998). At these residues, LpTFL1 differs from the consensus at one position (110), which is also the position with the highest degree of amino acid variation between species. It is interesting that the variation in amino acid residues at position 110 exactly matches the grouping of plant PEBP by the clustalW alignment (except for FT). One group comprising TFL1, BNTFL1-1, and' BNTFL1-3 has a Leu at this position, and another group comprising CEN, CET2, CET4, and SP has a Met, and a third group, which includes LpTFL1, FDR2, FDR1, and CET1, has a Ser at this position. The immediate assumption that the amino acid differences at this position can be linked to the variance in phenotype severity of plants overexpressing different *Plant PEBP* genes would suggest that overexpression of BNTFL1-1/BNTFL1-3, like TFL1, also has no effect in species like tobacco, and that over-expression of CET1, FDR2/FDR1, like LpTFL1, might have a significant effect on plant architecture and flowering time in species like Arabidopsis. Future results on overexpression of Plant PEBP genes in different species would contribute to clarify the correlation between protein sequence and the effect on morphology. In any case, our results show that the effects of different PEBPs cannot solely be explained by genetic diversity, since ryegrass is more distantly related to any of the dicot species, and yet LpTFL1 has a strong and unequivocal effect on the Arabidopsis.

***Control of Floral Transition***

The dramatic phenotype of Arabidopsis plants overexpressing *LpTFL1* suggests that in ryegrass, *LpTFL1* may play a role in controlling meristem identity and in the transition from vegetative to reproductive growth. In ryegrass, *LpTFL1* message is detected at all stages from germination to maturity. It is found at the apex, in the inflorescence, and also in leaves, stems, roots, and mature flowers. However, expression of *LpTFL1* in the ryegrass apex is not constitutive. Levels of *LpTFL1* message changed during flower induction with a slight induction in the SAM after 12 weeks of vernalization, followed by a strong up-regulation during long day (LD) induction until the structures of the spikelets were visible. Unexpectedly, the upregulation of *LpTFL1* mRNA during LD induction was even higher in the leaves (more than 25 fold). An increased upregulation of plant PEBPs in the leaves during LD induction has not previously been reported and it strongly suggests that LpTFL1 may play an additional role outside of the SAM. Some of the Arabidopsis

plants overexpressing *LpTFL1* never flowered before senescence. Vegetative non-flowering Arabidopsis plants have also been obtained by combining mutations in *AP1, CAULIFLOWER (CAL),* and *FRUITFULL (FUL),* all three MADS-box genes (Ferrandiz et al., 2000). The SAM of the *ap1, cal, ful* triple mutant is arrested in the vegetative to $I_1$ phase, producing only cauline leaves with axillary meristems that in turn repeat this pattern forming "leafy" cauliflower along the main inflorescence (Ferrandiz et al., 2000). Similar cauliflower-like structures were not observed in our UBI::*LpTFL1* plants because the repeated formation of meristems was slower. However, an additional morphological characteristic of the UBI::*LpTFL1* plants was the high density of trichomes that covered the leaves and the SAM (Fig. 8). In Arabidopsis, disappearance of the trichomes from the adaxial surface of cautine leaves has been shown to be tightly linked to floral induction and in support for this observation, it was shown that *tfl1* leads to accelerated loss of adaxial trichomes in Arabidopsis (Telfer et al., 1997). In agreement with this observation we find that the expression of a Plant PEBP gene in Arabidopsis prevents the loss of adaxial trichomes. By this criterion the UBI::*LpTFL1* plants were less competent to flower compared with the triple mutant. The UBI::*LpTFL1* plants which flowered after an extended vegetative phase produced normal flowers .This observation suggests that a delayed, but otherwise normal expression of the floral organ identity genes has occurred. Therefore, the level of LpTFL1 activity can be decreased over time or additional factors override LpTFL1 function and ensure the proper transcription of meristem and organ identity genes. One possible factor is FT, which is able to up-regulate floral meristem identity gene like AP1 and *LFY* (Kobayashi et al., 1999; Samach et al., 2000).

***A Potential Molecular Mechanism for Determinate Plant Architecture in Perennial Ryegrass***

Perennial ryegrass and Arabidopsis represent two different forms of plant architecture: determinate and indeterminate, respectively. A molecular basis for indeterminate growth has been proposed for Arabidopsis (Bradley et al., 1996, 1997; Ratcliffe et al., 1998, 1999;) in which indeterminate plant architecture is correlated with expression of *TFL1* in the centre of the SAM. In this central region as well as in the uppermost layers of the SAM, TFL1 activity is capable of excluding the expression of *AP1* and *LFY,* and therefore the formation of a terminal flower.. In ryegrass, *LpTFL1* message is present in the apex at the vegetative stage. Upon LD induction the

expression of LpTFL1 is significant upregulation, which is similar to the up-regulation of *TFL1* observed in Arabidopsis when the plants enter the $I_1$ phase (Ratcliffe et al., 1999).However in addition to what has previously been reported for plant PEBPs, LpTFL1 is strongly upregulated in the leaves during LD induction. Cellular localisation of *LpTFL1* expression in the ryegrass apex awaits characterisation, but the present results suggest that ryegrass architecture may be the result of a restriction of *LpTFL1* expression in the centre of the SAM to the meristematic ridges of the ryegrass apex (the axillary meristems) and the leaves. Analysis of transgenic ryegrass overexpressing *LpTFL1* is in progress, and based on the data presented here we can speculate the following scenario for LpTFL-mediated control of floral transition and plant architecture: Shortly after germination, *LpTFL1* expression is established in the meristematic ridges of the apex to maintain the production of vegetative organs such as leaves and tillers. During vegetative growth, a basal level of *LpTFL1* and other flowering repressors, perhaps similar to *FLC* (Michaels and Amasino, 1999), are maintained to avoid precocious flowering before the winter season. During the winter vernalization period, levels of *LpTFL1* slightly increase in the apex reflecting an increasing floral competence. As the temperature increases and the photoperiods lengthen in spring, *LpTFL1* expression is up-regulated in the apex to promote lateral branching of the main axis. In this way, a maximum number of spikelets are produced. Expression of *LpTFL1* subsequently becomes progressively more restricted to vegetative tissues such as leaves, stem and root, and the ryegrass plant finishes its life cycle by the production of the last uppermost seed in the top spike.

## 2. OVEREXPRESSION OF LpTFL1 IN PERENNIAL RYEGRASS

### MATERIALS AND METHODS

Ryegrass plant growth conditions, genomic screening, and RNA/DNA analysis were all performed as for example 1 above, with the exception that leaf samples from transgenic ryegrass lines were harvested after 28 ds of secondary induction.

#### *Plant Transformation*

The vector for overexpressing *LpTFL1* was constructed in the following way: Vector pUC19 was digested with *PstI* and *EcoRI* and re-ligated. The cassette of pAHC27 (Christensen and Quail) containing the maize Ubiquitin promoter, its first intron (Ubi1I), the *UidA* gene, and the NOS

terminator, was then ligated into the *HindIII* site of the modified pUC19. The *UiA* gene was removed from the cassette by digestion with *SmaI* and *SstI* and following *pfu*-polishing of the *SstI* protruding ends, the vector was re-ligated. Finally, the coding region of LpTFL1 cDNA (Jensen et al., 2001) was ligated into the BamHI site of the vector to give pLPTFL1.

pLpTFL1 plasmid was introduced into Lolium perenne together with pAHC20 (Christensen and Quail, 1996) harbouring the Bar gene, which confers resistance to the herbicide BASTA®. For particle bombardment highly embryogenic callus induced from meristems or mature embryos was used. Two different ryegrass cultivars (ACTION and TELSTAR) and one propagated clone (F6) were used as source for the callus production. Isolated embryos and meristems were cultured on a MS-based ((Murashige and Skoog, 1962) callus induction medium (CM) containing 3 % sucrose, 4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 100 mg/l casein hydrolysate and 0.3 % (w/v) gelrite (Kelco) for 12-26 weeks in the dark at 23°C. Calli were maintained by subculturing every third week on fresh CM-medium. Prior to bombardment, an osmotic pre-treatment for 4 hours were given by transferring small calli (2-4mm) to a solid MS-based medium supplemented with 3 % sucrose, 3mg/l 2,4-D, 0.25 M sorbitol, 0.25 M mannitol and 0,3 % w/v Gelrite. Bombardment was performed with a particle inflow gun (Finer et al., 1992) according to the optimised protocol described by Spangenberg et al. (1995) with a few modifications: bombardment pressure was 8 bar and 300 µg gold particles 0.6 µm (Biorad) were coated with 0.6 µg plasmid DNA (pLPTFL and pAHC20 at a molar ratio of 2:1) according to Vain et al. (1993). The following day, calli were transferred to CM-medium supplemented with 2 mg/l bialaphos (Meiji Seika Kaisha, LTD, Tokyo) and grown at 23°C under 16 hrs light. Selection at three weeks interval was performed until vigorously growing callus was obtained. Putative transgenic plants were regenerated by transferring calli to hormone free medium RM (MS-medium containing 3% sucrose and 2 mg/l bialaphos). Rooted plantlets were transferred to soil and grown to maturity under greenhouse conditions.

***Screening for Stable Transformation***

Putative transgenic plants were sprayed twice (two successive days) with a 0.5 % solution of BASTA (Hoechst Schering AgrEvo A/S, Germany) supplemented with 0.1 % Tween 20. The number of herbicide tolerant plants was scored after one week.

***PCR Analysis***

Genomic DNA was isolated from leaves of primary transformants (T0 generation) by the DNeasy 96 Plant kit (Qiagen), and the presence of the *LpTFL1* transgene was determined by PCR using primer LP0 (5'-ATGTCTAGGTCTGTGGAGCCTC-3') in combination with primer MS8 (5'-ACCGGCAACAGGATTCAATCT-3') to give a 560-bp fragment. Approximately 0.3 µg of genomic DNA was used in each reaction.

***Real-Time RT-PCR Analysis***

For the detection of *LpTFL1* mRNA level in the transgenic ryegrass lines poly-$(A)^+$ mRNA was isolated from crude lysate of approximately 100 mg leaf tissue from one individual per line. Leaf material was harvested prior to the vernalization treatment. Twenty microlitre of dynabeads (DYNAL) were used for each mRNA isolation and all the mRNA was used in the reverse transcription. One *LpTFL1* primer, INS5' was used in combination with a NOS terminator primer, MS8 in the subsequent Real-time quantitative PCR reaction to amplify a 320 bp *LpTFL1* fragment from the first strand cDNA templates. Amplification of PCR products were performed and monitored by the Rotor-Gene (Corbett Research) system in which inclusion of the fluorescent dye SYBR green I dye (Sigma Chemicals) into the reaction mixture facilitates direct measurement of double stranded DNA after each PCR cycle. One microlitre of each RT-reaction were used as template in the PCR reaction together with dilutions of plasmids containing *LpTFL1* and *LpGAPDH* (100, 10, 1 and 0.1 pg). In order to normalise the PCR results relative to the initial template amount in each sample, a PCR was run in parallel on similar samples with the primers GAP5 (5'-CAAGGACTGGAGAGGTGG-3') and GAP3 (5'-TTGACTCGTTGTCGTACC-3') to amplify a 380 bp *LpGAPDH* fragment.

**RESULTS**

***Overexpression of LpTFL1 Represses Flowering in Ryegrass.***

Thirty six transgenic ryegrass lines were obtained by microprojectile bombardment. All lines were resistant to BASTA®. Plants regenerated from a single transgenic callus (generation $T_0$)

were designated as a "transgenic line". Thus, each transgenic line traced back to a different tissue culture and represented an independent transformation event. PCR analyses of transgenic ryegrass leaf DNA using the primers LP0 and MS8 (Fig. 9) showed that the entire LpTFL1 coding region in connection with the NOS terminator was present in 22 lines, giving a 61% co-transformation efficiency. In one line (24), an additional product of 750 bp was amplified by the PCR reaction. Such a product may be amplified by each of the single primers if the transgene had integrated into the genome in a tail-to tail manner. Alternatively, it indicates, that fragmentated transgene DNA has been dispersed in the ryegrass genome.

Following three months of vernalization, all transgenic lines (inclusive all the lines which were tested negative for presence of the *LpTFL1* transgene and the non-transformed control) were transferred to LD conditions for floral induction. The number of inflorescences varied among the transformed lines with the biggest variation observed between the cultivars. This variance was also observed in the co-transformation efficiency (Figure 16) and reflects how different the cultivars responded to the transformation event. 'F6' gave the highest co-transformation efficiency (78%) followed by 'ACTION' (75%) and 'TELSTAR' (54%). Among the plants, which were tested negative for the *LpTFL1* transgene, 'F6' also produced more flowers (12,3 ± 5,1) than the two other cultivars ('TELSTAR'; 6,0 ± 4,2) and ('ACTION'; 1,4 ± 1,2). 'TELSTAR' and 'F6' in general looked more vigorous than did the cultivar 'ACTION'.

Despite the differences in the number of inflorescences, all the flowering lines headed almost simultaneously with the control after five weeks in LD (not shown). Several lines, however did not flower at all. Of the 22 transgenic lines, which were tested PCR positive for the *LpTFL1* transgene, ten remained non-flowering during the flowering season (six months), whereas only two of the 14 PCR negative lines remained non-flowering.

Using real-time RT-PCR we tested, whether the reduction in inflorescence production was correlated with the level of expression of *LpTFL1* from the *UBI::LpTFL1* transgene. We could detect *LpTFL1* transgene expression in 16 of the 22 PCR positive lines (Fig. 10). In order to distinguish between the transgene and the endogenous *LpTFL1* a NOS terminator primer was used in combination with and internal *LpTFL1* primer in the real-time RT-PCR. Subsequent analysis showed that the endogenous *LpTFL1* mRNA level in the leaves was 100-fold less than the lowest

detected *LpTFL1* transgene mRNA level at the point of harvest (not shown). A very high transgene expression level was detected in several lines, and we observed a clear and very dramatic effect of the *UBI::LpTFL1* transgene when *LpTFL1* was expressed at high levels. Five of the six lines (31, 32, 34, 35, 36) in which we detected the highest *LpTFL1* expression did not flower, and nine lines (23, 26, 27, 29, 31, 32, 34, 35, 36) of the 16 *LpTFL1* overexpressing lines remained non-flowering throughout the season. Overexpression of *LpTFL1* did not cause any other morphological changes when compared to the wild-type.

**DISCUSSION**

In ryegrass, *LpTFL1* message is detected at all stages from germination to maturity. It is found at the apex, in the inflorescence, and also in leaves, stems, roots, and mature flowers. However, expression of *LpTFL1* in the ryegrass apex is not constitutive. Levels of *LpTFL1* message changed during flower induction with a slight induction in the SAM after 12 weeks of vernalization, followed by a strong up-regulation during long day (LD) induction until the structures of the spikelets were visible. Unexpectedly, the upregulation of *LpTFL1* mRNA during LD induction was even higher in the leaves (more than 25 fold). An increased upregulation of plant PEBPs in the leaves during LD induction has not previously been reported and it strongly suggests that LpTFL1 may play an additional role outside of the SAM.

The control of floral transition (ie. the transition from vegetative to reproductive growth) has been studied extensively, especially in *Arabidopsis*, and a number of key regulators have been identified (for a recent review, see Simpson *et al.* (1999)). Knowledge of the biological function of these regulators is derived either from mutant studies or from experiments in which these genes were constitutively expressed in annual plants such as *Arabidopsis*, *Antirrhinum*, or tobacco. Previous results showed that *LpTFL1* is a strong repressor of flowering in annual plants, such as *Arabidopsis* (Jensen *et al.*, 2001). By introducing *LpTFL1* into ryegrass under the control of the maize ubiquitin promoter, we tested whether constitutive expression of *LpTFL1* was capable of preventing or inhibiting flowering in a perennial plant. Thirty six transgenic lines were produced of which 22 were tested positive for the *LpTFL1* transgene. Flowering was markedly reduced among

the PCR positive plants, and ten lines (45%) remained non-flowering during the flowering season. In contrast, only two lines out of the 14 PCR negative lines (14%) were non-flowering (Fig. 10).

The level of *LpTFL1* expression was tightly linked to the control of the vegetative to the reproductive phase. However, there was no linear correlation between the level of transgene expression and the flowering time (heading date) as previously observed in *Arabidopsis* (Jensen *et. al.*, 2001), and the floral repression was more seen as reduction in inflorescence production as a delay in heading date. We could detect *LpTFL1* transgene mRNA in 16 of the 22 PCR positive lines (Fig. 10), and nine of these lines (56%) remained non-flowering. Expression of *LpTFL1* at high levels comparable to housekeeping genes such as *GAPDH*, in this case prevented heading in five out of six lines (Fig. 10, line 31-36). No meristem proliferation or stem elongation was observed in the non-heading lines, which indicates that the plants were arrested in the vegetative phase.

Possibly, the control of vegetative to reproductive phase shift in the transgenic ryegrass is mediated by an intracellular LpTFL1 threshold level above which the plants will remain vegetative. Below the threshold level, the plants will flower and LpTFL1 will mainly affect stem length and panicle branching and only secondly the heading date. Unlike Arabidopsis, perennial ryegrass does not flower unless provided with a sufficient cold treatment under the right conditions. Therefore, it is conceivable that either the level of floral repressors are higher in perennials such as ryegrass than in annual plants like *Arabidopsis*.

It has previously been suggested that vernalization could overcome the activity of 35S::TFL1 in *Arabidopsis* (Simpson et al., 1999). We found that LpTFL1 was both actively transcribed and functional after three months of vernalization in perennial ryegrass.

The effect of *LpTFL1* overexpression was independent of genotype. Three different genotypes were used in the experiment and even though they all responded differently to the transformation with respect to co-transformation efficiency (Figure 16), the percentage of non-flowering *LpTFL1* overexpressing lines were equally distributed among them; ACTION, 55%; TELSTAR, 50%; and F6, 60%.

## 3. EXPRESSION OF LpTFL1 IN RED FESCUE

### MATERIALS AND METHODS

***Plant Transformation***

The pLPTFL1 plasmid was introduced into red fescue together with pAHC20 (Christensen and Quail, 1996) harbouring the Bar gene, which confers resistance to the herbicide BASTA®. Friable, embryogenic calli, ready for particle bombardment were prepared by growing excised embryos on a MS (Murashige and Skoog, 1962)-based callus-induction medium (MS5) for 10-12 weeks at 25°C in the dark. The MS5 medium was supplemented with 5 mg/l 2,4-dichlorphenoxyacetic acid (2,4-D), 500 mg/l casein hydrolysate and 3% (w/v) sucrose and solidified with 0.3% gelrite. Prior to bombardment, tissue pieces (3-4 mm) were transferred for osmotic pre-treatment in liquid medium containing 30g/l sucrose, 3mg/l 2,4-D, 0.25 M sorbital and 0.25 M mannitol for 30 min, and then transferred to the same medium solidified with 0.3% gelrite and incubated overnight in the dark. Gold particles (1.0 µm), coated with 12 µg of a mixture of pLPTFL1 and pAHC20 at a molar ratio of 1:1 were used for particle bombardment with a Bio-Rad PDS-l000 He Biolistic device (Biorad, Hercules, California) at 1300 Psi. Following bombardment, calli were placed on MS5 medium supplemented with 2 mg/l bialaphos (Shinyo Sangyo Ltd., Japan) and grown at 25 ± 1°C under 16 hrs light. After four to five successive rounds of selection at three weeks interval, putative transgenic plants were regenerated from the calli by supplementing the selection medium with 0.2 mg/l kinetin. Each callus tissue gave between one to four explants, which were transferred to soil and grown to maturity under greenhouse conditions.

***Screening for Stable Transformation***

All plants, including two non-transformed lines and two lines transformed only with the pAHC20, were screened for phosphinothricin acetyl transferase activity both by the Chlorophenol Red (CR) assay, and by their ability to withstand repeated applications of 5000 ppm BASTA® sprayed onto the foliage. For the CR assay, three to four healthy leaf tips from each plant were incubated on half-strength MS containing 8 g/l agar and 25 mg/l chlorophenol red with or without 8 mg/l bialaphos. Leaves from non-transgenic plants were distinguished from the putative transgenic

plants by the development of severe necrosis associated with a characteristic red coloration of the medium.

After six months of growth, plants from each line were vernalized either artificially or under natural field conditions (winter 2000-2001, Denmark). For the artificial vernalization, plants were kept in a growth chamber at or below 5°C for 21 weeks. During vernalization, the light period was decreased to 8 hrs per day. Following vernalization, all plants were transferred to long day (LD) conditions (16 hrs light at 22 and 20°C, day and night temperature, respectively) for floral induction. After seven months, the clones were cut back and submitted to the second round of floral induction, which included vernalization under natural field conditions (winter 2001-2002) and the subsequent growth in summer conditions. The number of culms with seed head from each individual clone was recorded in both flowering seasons and the length of the five longest inflorescences was measured during the first season.

***PCR and DNA Gel Blot Analysis***

Genomic DNA was isolated from leaves of primary transformants (T0 generation) by the FastDNA® ORANGE kit DNA isolation system (Bio 101), and the presence of the transgene was determined by PCR. Different primer combinations were used to examine the genomic integration and arrangement of the transgenic DNA (Figure 17).

The forward primers were MS31 (5'-CGTGGCGGAGCGGCAGAC-3'), MS33 (5'-TAGTACATCCATTTAGGGTTTAGG-3'), MS56 (5'-TATTTATTTGCTTGGTACTG-3') and LP0 (5'-ATGTCTAGGTCTGTGGAGCCTC-3'), and the reverse primers were LP4REV (5'-CGAACCTGTGGATACCAATG 3'), LP575 (5'-GGGATCCCACAACTGGGATAGCCAAGAACT-3') and MS8 (5'-ACCGGCAACAGGATTCAATCT-3').

Genomic DNA for the gel blot analysis was isolated from the leaves of one to three individuals of different transgenic lines by the Phytopure® Genomic DNA isolation system (Nucleon). DNA (10-30 µg) were digested overnight with restriction endonucleases *Hin*DIII and *Eco*RI (separately) and fractionated on a 0.8% agarose gel and blotted onto Amersham Hybond N membrane in 20% SSC according to the manufacturer's recommendations. Probe DNA generated by PCR using the primer set MS56-LP4REV on plasmid DNA (Figure 17) was radiolabeled with γ-

$^{32}$P-labelled dCTP (3,000 Ci/mmol) through the random primer method (Megaprime, Amersham). Pre-hybridisation, hybridisation and the subsequent washing steps were performed according to standard protocols.

***RNA Gel Blot Analysis***

Seventy five micrograms of total RNA were isolated from leaves of one to four clones from each transgenic line according to Sambrook *et al.* (1989). Purified poly-A$^+$ mRNA (Dynabeads, DYNAL, Norway) from one individual of each line was fractionated under denaturing conditions and transferred onto Hybond N membranes in 20% SSC. The membranes were hybridised to a 180 bp *LpTFL1* cDNA fragment and a 450 bp *ACTIN* cDNA fragment for standardisation. The other individuals from the transgenic lines were analysed in a similar way, using total RNA. Relative *LpTFL1* expression levels in the transgenic lines were estimated on the basis of the results from a density scan (Quantity One software, Biorad) of the autoradiograph in which the *LpTFL1* expression level in the highest expressing line (J) was set to 100.

**RESULTS**

Eighteen transgenic fescue lines were obtained by microprojectile bombardment. In addition, two lines (BAR1 and BAR2) were obtained by transformation only with the plasmid pAHC20. All lines were resistant to BASTA® and showed phosphinothricin acetyl transferase activity. Plants regenerated from a single transgenic callus (generation $T_0$) were designated as a "transgenic line". Thus, each transgenic line traced back to a different tissue culture and represented an independent transformation event. PCR analyses of transgenic fescue leaf DNA using the primers MS56 and LP4REV (Figure 17) indicated that *LpTFL1* was present in 14 lines, giving a 77% co-transformation efficiency. These 14 lines (A-N) together with BAR1, BAR2 and two non-transformed lines were selected for further characterisation.

***Transgene Integration***

The DNA from the transgenic plants was digested with *Hin*DIII, which released a 2.8-kb fragment containing the ubiquitin promoter and the *LpTFL1* coding region (Figure 17). Restriction

patterns of transgenic DNA were complex in several lines (Fig. 11). Restriction fragments of the expected size were found in four lines (D, I, J, and L, Fig. 11A and not shown). All lines contained fragments larger or smaller than the expected size, which represented rearrangements of the transgene DNA. There were no rearranged fragments of the same size recurrently observed in different lines (Fig. 11A) except for a 2.1 -kb fragment, which was also present in the controls and may correspond to the endogenous *F. rubra TFL1*-like *(FrTFL1)* gene. Faint or smeared signals were also detected in restricted DNA from BAR1 and BAR2 (Fig. 11), which may represent the plasmid pAHC20 that carries the same ubiquitin promoter:exon:intron construct to drive *Bar* expression.

DNA from transgenic plants was also digested with *Eco*RI which has only one restriction site in the vector at the 3'-end of the NOS terminator and was expected to yield fragments corresponding in size to the repeats in a pALPTFL1 concatamer if plasmid concatenation had occurred. Multiple different-sized *Eco*RI restriction fragments hybridising to the *intron::LpTFL1* probe (Fig. 11B) indicated that concatenation of full-length plasmid copies was not the predominant mode of transgene organisation in the plant genome. Two lines (D and I) contained fragments of the expected size (5.5 kb), however the subsequent attempt to PCR amplify the transgene promoter in these lines failed (see below). It was difficult to determine the exact transgene copy number especially because several lines contained truncated plasmid copies. Nevertheless, we estimated it to vary from two (line G) to twenty (line D).

Long PCR using different primer combinations to amplify parts of the ubiquitin-exon-intron-LpTFL1-nos cassette (see Figure 17) was performed to examine if the transgenic lines contained intact cassettes or if transgene rearrangement had occurred internally in this region. The 3'-end of the cassette containing the *LpTFL1* coding region and the NOS terminator appeared to be intact in all lines except for A and F (Figure 17). In addition to the fragments of expected size (0.6 kb), a 0.5 kb fragment was detected in three lines D, G and N, when PCR was performed with the primer set MS56 and LP575. When PCR was performed with the primers LP0 and MS8 we detected fragments larger than the expected size in lines D, I and N. Such fragments may be amplified by each of the single primers if the transgene had integrated into the genome in a tail-to-tail manner. Alternatively, fragmented transgene DNA may have been dispersed in the fescue genome. Smaller

fragments must reflect DNA deletions, and since these fragments were only detected when using the primers MS56-Lp4REV and not LP0-MS8, the deletion is likely located in the 3'-end of the *ubiquitin intron*.

The promoter part of the UBI::LpTFL1 cassette was analysed by PCR using two primers (MS33 and MS31) located 500 and 100 bp upstream the TATA box, respectively, in combination with primers matching the LpTFL1 coding region. The results schematically described in Figure 17, revealed that two lines (J and L) contained the full-length promoter, while 6 lines only contained a short partial ubiquitin promoter (including the MS31 primer site). In line B, C, E, F, I and K the promoter part was either absent or dispersed and/or reoriented from the LpTFL1 coding region, and in line D and N a 1.5-kb DNA fragment had been deleted between MS33 and LP4REV (including MS31 and the TATA box). A 100 bp deletion was also found in the promoter of lines G, and although the exact location was not determined we assume it to be close to the 3'-end of the UBI intron. The intron part was found to be intact in line A, D, G, H, J, L and M but not in line B, C, E, F, I, K and N (MS31-LP4REV). In all, two lines (J and L) were found to contain at least one complete expression cassette (Ubi-ex-intron-LpTFL1-nos).

***LpTFL1 Expression Represses Flowering.***

All transgenic T0 plants grew normally and their morphology did not deviate from the wild-type controls during the vegetative stage (not shown). The morphological differences between the wild-type and the transgenics became apparent after plants had been induced to flowering. The wild-type and the BAR1, BAR2 plants flowered approximately two weeks before the UBI::LpTFL1 plants. The only exception was the plants from line A, which flowered simultaneously with the wild-type. There was a clear difference in the flowering response between the plants, which were vernalized artificially and those, which were vernalized under field conditions (winter 2000-2001). While only two of the artificially vernalized UBI::LpTFL1 lines flowered, ten of the naturally vernalized lines flowered (not shown). Notably, except for one plant, all the artificially vernalized control and BAR1, BAR2 plants flowered (not shown). This observation indicates that although the conditions during the artificial vernalization were sufficient to allow the subsequent flowering induction, the UBI::LpTFL1 lines required stronger environmental stimuli, which were only present

under field conditions. For the subsequent analysis of the LpTFL1 transgene effect, we decided to base our results only on the data from the naturally vernalized lines.

The number of inflorescences produced by each clone during the first season varied markedly between the lines (from 0 to 138, Figure 12A). Fewer inflorescences were produced the second year because the clones were divided into smaller units. Stem (culm) length also varied between the lines (Fig. 10B), and it did not change significantly from the first to the second flowering season (not shown). Four UBI::LpTFL1 lines (K, L, M and N, Figure 12 A and B) did not flower during the seven months following the first vernalization, and three of these lines (K, L, and M) also remained non-flowering during the second season. Two lines (I and J) produced only a single flower from three individual clones during the first season and only one and three flowers during the second season, respectively.

RNA gel blot analysis was performed to test whether the reduction and delay in inflorescence production was correlated with the expression of LpTFL1 from the UBI::LpTFL1 transgene. The level of LpTFL1 message varied from zero to levels comparable to ACTIN mRNA (Fig. 13). Three of the four lines (K, L, M) in which the highest LpTFL1 expression was detected did not flower, and the fourth line (J) produced only 0.3 inflorescence per clone (Fig 12A and 14). Lines with a lower level of LpTFL1 message produced flowers, and there was a trend (although not statistical significant with the present material) towards a reduction in the number of inflorescences per clone with increasing levels of LpTFL1 mRNA (Fig. 12A).

No LpTFL1 message was detected in line D, F and N (Fig. 12 and 13). This finding correlated well with the observation that the transgenes in these lines either lacked the UBI promoter (line F) or had a partial UBI promoter lacking the TATA box (line D and N, Figure 17).

The LpTFL1 message in line A was 80-120 bp smaller than expected (Fig 13). We propose that this fragment represents a truncated LpTFL1 transcript, which is overexpressed in this line. This assumption is strengthened by the fact that, for this line, we were unable to PCR amplify the LP0-MS8 fragment, which contains the LpTFL1 coding region and the NOS terminator (Figure 17). In addition, we found that plants from line A flowered simultaneously with the wild-type and produced the highest average number of inflorescences among all the UBI::LpTFL1 lines (Fig. 12A). Line A plants were also among the tallest plants included in the investigation (Fig 12B), and

they produced panicles, which were generally reduced in size compared to the control (Fig. 15). Oppositely, the single flowering plant of the high expressing line J, was the shortest of all flowering plants (Fig. 12A). However, with the present data in hand, there is no statistical significance to confirm a correlation between the level of LpTFL1 expression and culm length.

**DISCUSSION**

Eighteen Basta® resistant red fescue lines were obtained by particle bombardment. Plants from fourteen different lines were tested positive for the gene of interest by PCR. DNA gel blot analysis of different lines revealed that the transgene had integrated into the fescue genome in a complex fashion and that multiple transgene rearrangements had occurred. Transgene rearrangements included deletions in the promoter regions and in the LpTFL1 gene (Figure 17). Highest expression of LpTFL1 was detected in plants containing the full UBI::LpTFL1 cassette. Deletion of promoter sequence lead in most instances to a reduction in LpTFL1 expression compared to the high expressing lines (Figure 17 and Fig. 12). Expectedly, if the deletion included the TATA box, the partial promoter was defect and no LpTFL1 transcripts could be detected in the plants. However, one line (K) expressed LpTFL1 at high levels although we could not PCR amplify any fragments corresponding to the UBI promoter construct in this line. There is no obvious explanation for this observation, but either none of the three PCR reactions worked or, alternatively, parts of the cassette could have integrated into a transcriptionally active region.

Analysis of the transgenic lines showed that LpTFL1 expression in red fescue is tightly linked to the control of vegetative to reproductive phase shift. All lines containing the LpTFL1 transgene, except line A (discussed below), flowered at least two weeks later than the wild-type and the BAR controls. However, there was no linear correlation between the level of transgene expression and flowering time (heading date) as was previously observed in Arabidopsis (Jensen et al., 2001). Expression of LpTFL1 at high levels comparable to housekeeping genes such as ACTIN, in this case prevented heading in three out of four lines and in the fourth line only one inflorescence was produced within three clones (Fig. 12A and 14). At moderate expression levels, LpTFL1 expression caused a general reduction in the number of inflorescences and in the stem

length but an increase in panicle branching, although the statistical significance of these observation requires a more thorough investigation of the second generation plants. . Similar observations were made by Nakagawa et al. (2002) in the analysis of transgenic rice overexpressing RCN1/2. They found that constitutive expression of RCN1/2 at moderate levels were associated with a three-fold increase of secondary branches and even production of tertiary branches, which is not seen in wild-type rice. Expression of RCN1/2 at high levels led to stem retardation and a 'never-heading' phenotype. However, the 'never-heading' plants still produced a flag leaf and an immature panicle, enclosed by leaves, indicating that the transition from vegetative to reproductive phase finally took place (Nakagawa et al., 2002). These results are in contrast to our observations, which show that the 'never-heading' red fescue plants presented here, are arrested in the vegetative phase, since they do not produce stems or panicles.

Possibly, the control of vegetative to reproductive phase shift in the transgenic red fescue is mediated by an intracellular LpTFL1 threshold level above which the plants will remain vegetative. Below the threshold level, the plants will flower and LpTFL1 will mainly affect stem length and panicle branching and only secondly the heading date. This correlation is in contrast to the results in Arabidopsis, where the flowering date was linearly correlated to the level of LpTFL1 expression (Jensen et al., 2001), and it may reflect the difference in vernalization response between the two species. Both Arabidopsis and red fescue are sensitive to vernalization. However, unlike Arabidopsis, red fescue does not flower unless provided with a sufficient cold treatment under the right conditions. Therefore, it is conceivable that either the level of floral repressors is higher in red fescue than in Arabidopsis or, that the non-flowering threshold level is lower. It has previously been suggested that vernalization could overcome the activity of 35S::TFL1 in Arabidopsis (Simpson et al., 1999). We found that LpTFL1 was both actively transcribed and functional even after two rounds of natural vernalization in red fescue.

The molecular function of TFL1-like proteins is not known. Although a putative nucleotide-binding region in TFL1 has been described (Ohshima et al., 1997), recent data suggest that TFL1-like proteins more likely may interfere with regulatory kinase cascades (Banfield and Brady, 2000). Future molecular analysis should concentrate on identifying genes, which are either up- or downregulated in the transgenic fescue lines expressing LpTFL1 at high levels.

Despite the fact that no LpTFL1 expression was detected in line N, these plants remained non-flowering during the first season. It is most likely that this deviation from the other results can be ascribed to the carry-over effects from the tissue culture, since this line started to produce flowers during the second season (Fig. 12A).

The phenotype of the transgenic line A was reminiscent of a putative weak Festuca rubra tfl1-like( frtfl1) mutant phenotype. In this line, culm and panicle formation was favoured at the expense of decreased leaf production (Fig. 14), and the panicles were more compressed and wrinkled than the wild-type (Fig. 15). Line C plants in contrast, produced panicles, which were generally larger and contained more spikes with more spikelets than the wild-type panicle. Line C plants expressed LpTFL1 at a relatively high level, suggesting that the increased branching is a direct effect of increased levels of LpTFL1. Consistent with this hypothesis is the assumption that the decreased branching observed in line A is caused by a C-terminal truncation of the LpTFL1 protein. Interestingly, it was recently found that the function of the proteins belonging to the TFL1 family in Arabidopsis is dependent on the C-terminal part of the protein. FLOWERING LOCUS T (FT), which is very homologous to TFL1, but acts oppositely (Kardailsky et al., 1999), is mainly determined by the C-terminal part of the protein. By swapping exons between the FT and TFL1 cDNAs, Ahn and Weigel, (2001) found that the last exon distinguishes between FT- and TFL1-like properties of the chimeric gene.

In line with our interpretation of the data concerning the line A plants, He et al. (2000) recently reported that overexpression of the Arabidopsis LFY from the 35S CaMV promoter lead to early heading in rice. However, early flowering was accompanied by a reduction in grain yield, which was ascribable to smaller panicles containing 7-9 fewer seeds. In addition, He et al. (2000) found that transgenic 35S::LFY plants had on average one or two leaves less than wild-type plants. It has previously been shown that 35S::LFY Arabidopsis plants are reminiscent of the tfl1-mutants (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995) and that mutation in TFL1 leads to ectopic expression of LFY and other floral meristem identity genes in the shoot apex (Bowman et al., 1993; Bradley et al., 1997). Based on these results, we find it reasonable to suggest that the transgenic fescue line A plants are phenotypically reminiscent of the rice 35S::LFY plants and thus may represent a fescue tfl1-like mutant. Analysis of the T1 generation for co-segregation of panicle

branching with the UBI::LpTFL1 transgene will reveal if the suggestion is true, and the isolation of a LFY-like (or an AP1-like) gene from red fescue will allow us to determine if the expression level of these floral identity genes are increased in the UBI::LpTFL1 line A plants.

Our results show that expression of the heterologous LpTFL1 in red fescue at high levels can prevent flowering (Fig. 12A and 14). Additionally, it appears that the level of LpTFL1 expression in flowering plants may cause a reduction in culm length (Fig. 12B) and leaf width (not shown), although this needs to be further examined. No other morphological effects of the transgene expression were observed.

Implementation and utilisation of a LpTFL1-mediated non-flowering phenotype into commercial breeding strategies will require a mechanism, by which the LpTFL1-mediated floral repression can be relieved. Such a mechanism could be provided by combining LpTFL1 expression with the expression of a floral activation gene from a promoter, which can be activated at any time by application of the appropriate ligand.

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